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Degree: Master of Science

Year this Degree Granted: 2001

University of Alberta

REQUIREMENT FOR MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN IN THE ASSEMBLY OF APOB-CONTAINING LIPOPROTEINS

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science in Experimental Medicine

Department of Medicine

Edmonton, Alberta

Fall 2001

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Requirement for Microsomal Triglyceride Transfer Protein in the Assembly of ApoB-containing Lipoproteins" submitted by Agnes Kulinski in partial fulfillment of the requirements for the degree of Master of Science in Experimental Medicine.

Abstract

The assembly of Very Low Density Lipoproteins (VLDL) is dependent on Apolipoprotein B (apoB), Microsomal Triglyceride Transfer Protein (MTP) and lipids. MTP, based on *in vitro* studies, transfers lipids between membranes. However, the physiological role of MTP in the assembly and secretion of VLDL in the liver is not fully understood.

We hypothesize that MTP is required for the mobilization and transfer of lipids to apoB and the lipid droplet residing in the lumen of ER. To test our hypothesis, we used an MTP inhibitor (BMS-197636 02). In the first approach, we isolated mouse hepatocytes and used them to label apoB and lipids with different radioisotopes to follow their metabolism in the presence or absence of MTP inhibitor.

Our results suggest apoB can be translocated across the membrane of ER in both MTP inhibitor-treated and control hepatocytes. The density fractionation of newly assembled lipoproteins isolated from the lumen of ER and secreted apoB-containing lipoproteins showed that in MTP inhibitor treated hepatocytes, VLDL levels were decreased significantly or were absent. The levels of poorly-lipidated, apoB-containing lipoproteins increased. We also found that the inhibitor abolished the secretion of apoB100 and the levels of apoB48 secreted were also affected, but at a higher concentration of MTPI. Lipid studies showed decreased recovery of lumenal apoB-associated TG as well as TG in the lumenal lipid droplet in cells with reduced MTP activity. In MTPI treated cells the

secretion of TG decreased by approximately 80%, and was associated with apoB48-containing particles.

We conclude MTP is able to mobilize and transfer TG into the lumen of ER for apoB-containing lipoprotein assembly and the formation of lumenal lipid droplet.

Acknowledgements

I wish to thank my supervisor, Dr. Jean Vance for her guidance and support. She was always there to provide me with help and at the same time she gave a complete freedom with my work and trying new ideas.

I am very grateful to Dr. Richard Lehner, Dr. Sabina Rusteaus and Anna Noga for offering me their expertise in the field of apoB.

I extend my thanks to the members of the JV lab for all their help, friendship and advise on how to operate a Macintosh computer: Russell Watts, Benedicte Balcerzak, Paul Grandmaison, Sherri Selby, Barbara Karten, and Igor Cvetkovic.

I would like to acknowledge my committee members Dr. Hobman, Dr. Lehner, and Dr. Thomson, for helpful suggestions with regards to my research.

I also thank Russ Watts and Jody Aldrich for the technical support and mice surgeries.

I am thankful to Dr. Donna N. Douglas, Dr. Jean E.Vance, Dr. Paul A. Grandmaison and Sherri Selby for revising my thesis, their criticism and insightful comments.

Of course I would like to thank my friends from outside of my work for stopping me from spending too much of my free time in the lab and to take pleasure in what Edmonton has to offer.

Dedications

I dedicate this thesis to my parents and my brothers

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Abbreviations

a.a.	amino acid
ABL	abetalipoproteinemia
ALLN	N-acetyl-leucyl-leucyl-norleucinal
apo	apolipoprotein
APOBEC-1	apoB editing complex-1
BSA	bovine serum albumin
CE	cholesteryl ester
Ci	curies
COS	CV-1 origin, SV40 transformed monkey kidney cell line
C-terminus	carboxyl-terminus
d	density
DG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DPM	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
ER	endoplasmic reticulum
FFA	free fatty acid
g	grams
KO	"knockout"
l	liters

LDL	low density lipoprotein
LV	lipovitellin
McA-RH7777	McArdle rat hepatoma 7777
M	moles/liter
MTP	microsomal triglyceride transfer protein
N-terminus	amino-terminus
PA	phosphatidic acid
PBS	phosphate buffered saline
PC	phosphatidylcholine
PDI	protein disulfide isomerase
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PL	phospholipid
PMSF	phenylmethanesulfonyl fluoride
PS	phosphatidylserine
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS PAGE	SDS polyacrylamide gel electrophoresis
SUV	synthetic small unilamellar vesicles
TG	triacylglycerol
TGH	triacylglycerol hydrolase
TLC	thin layer chromatography
VLDL	very low density lipoprotein

A	adenine
C	cytosine
G	guanine
T	thymine
U	uracil

Aln	alanine (A)
Asn	asparagine (N)
Asp	aspartic acid (D)
Arg	arginine (R)
Cys	cysteine (C)
Glu	glutamic acid (E)
Gln	glutamine (Q)
Gly	glycine (G)
His	histidine (H)
Ile	isoleucine (I)
Leu	leucine (L)
Lys	lysine (K)
Met	methionine (M)
Phe	phenylalanine (F)
Pro	proline (P)

Ser	serine (S)
Thr	threonine (T)
Trp	tryptophan (W)
Tyr	tyrosine (Y)
Val	valine (V)

Chapter 1 - Introduction

1.1 Introduction

Heart disease is the major cause of death in Western countries. The primary cause is the deposition of Low Density Lipoprotein (LDL)-derived cholesterol on the arterial walls. These lipoprotein particles arise from the initial assembly of Very Low Density Lipoproteins (VLDL) in the liver. Subsequently, VLDL is secreted into the plasma and a complex series of enzymatic reactions converts VLDL to LDL. The primary function of the lipoproteins is to deliver lipids such as triacylglycerol (TG), cholesterol and cholesterol ester to the peripheral tissues. The lipid composition of the lipoprotein depends on the diet of the individual and the proper function of all enzymes, receptors and ligands that determine the lipoprotein metabolism. Great effort has been invested into understanding the molecular mechanisms that control assembly and secretion of lipoproteins. The accomplishments have already assisted in the development of effective therapeutic agents, which lower plasma LDL and ultimately reduce the mortality associated with heart disease.

The major requirements for VLDL assembly were established by studying genetic disorders that manifest as a decrease or elimination of plasma VLDL. Hypoabetalipoproteinemia is a genetic disorder that is characterized by the absence of apoB-containing lipoproteins from the plasma (Aguie *et al.*,1995). Studies with patients having this disorder led to the discovery of a major protein

component of VLDL, namely apolipoprotein B (apoB), a protein which spans the lipoprotein coat. Similar symptoms are observed in abetalipoproteinemia (ABL), a disease in which Microsomal Triglyceride Transfer Protein (MTP) gene is mutated. Although MTP itself is not a part of the lipoprotein, it is thought to facilitate the addition of lipids to apoB. Both the mandate and mechanism of MTP have not been fully elucidated and these are currently subjects of great controversy between different research groups. The opinions and theories put forward after over two decades of investigations are summarized in this chapter.

1.2 Structure of apoB

Full-length apoB is a 4536 amino acid (513 kDa) secretory glycoprotein synthesized in the liver. This protein is heavily modified and besides 20 potential N- and O-linked glycosylation sites it contains a relatively large N-terminal signal sequence. Overall, the protein has 25 cysteine residues of which 16 form intramolecular disulfide bonds (Tran *et al.*, 1998), as well as phosphorylation and fatty acylation sites (Hoeg *et al.*, 1988). These features give the protein a complex tertiary structure which facilitates the tight packaging of lipids within the core of the lipoprotein particle.

The intestine produces apoB 48 which is N-terminal 48% of full length apoB and was found to be encoded by the same gene edited at the mRNA level through the action of enzyme known as apoB editing complex-1 (APOBEC-1) (Davidson & Shelness, 2000). ApoB 48 is a result of premature termination of

apoB mRNA at position 6666 where conversion of base C to U by APOBEC-1 occurs and the codon encoding glycine is converted to a stop codon. Unlike primates which synthesize apoB100 only, the liver of rodents produces both forms of apoB, apoB100 and apoB48.

ApoB assumes a pentapartite structure in which three amphipathic α-helical structures, α_1, α_2, α_3, alternate with two antiparallel amphipathic β strands, β_1 and β_2 (Segrest *et al.*, 1994) as shown in Fig. 1.1. α_1 is the NH_2-terminus of the protein consisting of 14 cysteine residues that form disulfide bonds resulting in a globular domain. The rapid disulfide bond formation drives hydrophobic domains, or adhesion zones, into the interior of the protein, a step that prevents exposure of hydrophobic surfaces that may cause intracellular aggregation (Yilla *et al.*, 1992). α_2 and α_3 are distributed in two clusters located between amino acids 2100-2700 and 4100-4500 and are thought to confer strong but reversible binding of apoB to the lipoprotein surface. The β strands are more substantial and make up a greater portion of the peptide. The β_1 strand is located between B18.2 and B43.2 (N-terminal 18.2% and 43.2% of full length apoB) and β_2 between B55.6 and B85.3 (N-terminal 55.6% and 85.3% of full length apoB). These regions may be responsible for the irreversible integration of apoB with the triacylglycerol (TG) core of the lipoprotein particle. The pentapartite structure permits apoB100 to permanently weave in and out of the lipoprotein core.

The association of apoB on the lipoprotein surface was mapped using electron microscopy and 11 monoclonal antibodies to different apoB epitopes (Chatterton *et al.*, 1991), (Chatterton *et al.*, 1995); as presented by Fig.1.2. It has

been shown that first 89 % of apoB wraps around the lipoprotein like a belt closing the loop at residue 4050. The remaining C-terminal 11% forms a bow crossing over the belt structure around residues 3000-3500, which is near the suggested LDL receptor binding site. It has been presented that the bow structure may function as a negative regulator of LDL receptor binding, thus being responsible for the clearance of LDL from plasma (Boren *et al.*,1998).

The very involved structure of this enormous protein and the process of integrating it with lipids to assemble VLDL suggest the necessity of other means that would provide help in this demanding and time consuming process. ApoB interacts with multiple ER-resident molecular chaperones during its maturation (Linnik & Herscovitz, 1998). Those chaperones may differ between cell lines used to study the interactions. Co-immunoprecipitation experiments showed physical interaction of apoB with calnexin, ER60/ER57, BiP/GRP78, GRP94, caleticulin, ERp72 and MTP (Mitchell *et al.*, 1998). The absence of any of the chaperones causes a prolonged interaction with the translocon or the accumulation of apoB on the inner membrane of ER. At this point apoB becomes subject to degradation, which occurs at two different sites; a lumenal and cytosolic site, where different proteases and a proteosome degrade unwanted apoB (Fisher *et al.*, 1997).

1.3 Microsomal Triglyceride Transfer Protein

MTP is a heterodimeric protein found predominantly in the liver hepatocytes and intestinal enterocytes. It consists of a large 97 kDa subunit that was recognized for its *in vitro* lipid binding ability and their transport between membranes. A small 55 kDa subunit was previously identified as Protein Disulfide Isomerase (PDI) and is responsible for disulfide bond formation and chaperoning (Wetterau *et al.*, 1991a). The irreversible interaction with PDI maintains the larger subunit of MTP in a soluble form and is essential for the proper structure and activity of MTP (Wetterau *et al.*, 1991b). PDI may also be responsible for targeting MTP to its final destination, the ER lumen, since an ER retention sequence (KDEL) is present on PDI but not on MTP (Wang *et al.*, 1997a).

1.3.1 Lipid transport properties of MTP

In vitro, MTP binds and shuttles lipids, both neutral and phospholipids, between membranes (Atzel & Wetterau, 1993). MTP lipid transfer activity is determined by measuring the rate of transfer of radiolabeled TG from donor synthetic small unilamellar vesicles (SUV) to acceptor SUV. Donor vesicles contain PC, radiolabeled TG and cardiolipin which gives the donor vesicle a negative charge to facilitate the separation from acceptor vesicles. The acceptor vesicle contains a larger amount of PC and some unlabeled TG. When there are

equal amounts of TG in the donor and acceptor membranes, MTP promotes the exchange of TG between membranes. In the presence of different lipid composition of donor and acceptor vesicles, MTP is capable of net TG and CE transport (Atzel & Wetterau, 1993).

The C terminus of MTP is responsible for the binding and transfer of lipids with a stoichiometry of 1-5 lipid molecules per subunit (Atzel & Wetterau, 1993). MTP forms a stable complex with a variety of lipids such as TG, cholesterol ester (CE), diacylglycerol (DG), squalene, phosphatidylcholine (PC), phosphatidylethanolamine(PE), phosphatidylserine (PS), phospatidylinositol (PI) and phosphatidic acid, with a preference for neutral lipids. The ability of MTP to bind lipids at the membrane surface is related to the concentration of lipids in the donor membrane and the size of the hydrophobic moiety, regardless of the lipid class. The size of the hydrophobic moiety of the transferred lipid has been shown to influence the rate of transport by MTP. For example, the addition of a fatty acyl chain or chains to monoacylglycerol (MG), cholesterol, or lyso-PC dramatically increases their transfer rates. Lipid transport rates have been shown to decrease in order of TG>CE>DG>PC (Atzel & Wetterau, 1993)

It is interesting that varying head group charges of phospholipids do not change the rates of transport, suggesting that there are no specific, structural or charge-charge interactions between MTP and the head group of a phospholipid. Therefore, MTP is quite flexible in the nature of the molecules it can bind, suggesting there are minimal specialized interactions between the lipid molecules and MTP.

Other enzyme kinetic studies have shown that MTP contains two classes of lipid molecule binding sites (Atzel & Wetterau, 1994) a fast site(s) which rapidly transports both neutral lipids and phospholipids, and a slow site(s) which appears to be selective for phospholipids, slowly transferring them between membranes. It is still uncertain how MTP transfers lipids *in vivo*.

1.3.2 Modelled structure of MTP

MTP displays amino acid sequence homology with lipovitellin (LV), an ancient protein found and characterized in egg-laying animals. LV is responsible for the lipid storage and transport to the yolk sac (Mann *et al.*, 1999). It was shown that the predicted secondary structure of LV is consistent with the molecular structure of MTP. The high degree of conservation between their secondary structure suggests that their tertiary structures would be similar as well. X-ray crystallography of LV depicts a globular amino-terminal β-barrel made up by 13 β-strands, an α-helical domain comprised of 17 helices and a carboxyl-terminal lipid-binding cavity lined by two β-pleated sheets as shown in fig. 1.3. The surfaces of the outer helices are used in homodimerization. The large lipid cavity, a presumed location of the non-covalently bound lipids, has the shape of a cone with walls of the cavity comprised of β-structure with only limited α-helical structure located in the areas in which interactions with lipids are possible. The cavity seems to be separated from solvent by a cap, which may serve to stabilize the lipids in this region. It has been suggested that the cavity is flexible enough to

allow its volume to adjust according to the number of lipids sequestered (Raag *et al.*, 1988).

The tertiary structures of MTP and apoB were approximated using database searches and phylogenetic analysis because of the challenge to crystallize large proteins with variable lipid content. Based on the primary sequence homology among LV, MTP and apoB, it has been suggested that MTP and apoB adopt the predicted structure of LV and share the same functional characteristics. The major difference between these proteins is found in their different lipid binding properties (Mann *et al.*, 1999) as they bind different amounts and kinds of lipids. LV binds phospholipids with a stoichiometry of 35 molecules/subunit (Timmins *et al.*, 1992), MTP binds 1-5 molecules of lipid (Atzel & Wetterau, 1993), whereas apoB incorporates a large neutral lipid core (Segrest et al., 1994). The amino acid residues 22-300 of MTP and amino acid residues 1-290 of apoB resemble the β-barrel structure of LV (Fig. 1.4). The predicted α-helical domains of MTP (amino acid residues 304-598) and apoB (amino acid residues 294-592) consist of 17 helices arranged in an inner and outer layer (Fig. 1.4). As in LV, the central portions of the helical structures are stabilized by a disulfide linkage. Amino acids 613-826 of MTP are thought to form a lipid binding cavity which is smaller than the cavity found in LV (Fig. 1.5). The predicted lipid binding surfaces of this MTP model contain a number of hydrophobic residues comparable to those found on the equivalent surface of LV, providing evidence for the validity of the proposed MTP structure. The back of the cavity of MTP may be closed off by dimerization with PDI, since the posterior

opening to the lipid binding cavity of LV is closed off by homodimerization. The buried salt bridge of LV is also likely present in both MTP and apoB, since the same arginine residue is conserved. These structural similarities suggest that LV, MTP and apoB, function in a similar fashion to transfer lipids.

1.4 A mechanism of membrane lipid acquisition by MTP

Read *et al* have suggested a mechanism of membrane neutral lipid acquisition by MTP for transfer of lipids to apoB during lipoprotein assembly (Read *et al.*, 2000). MTP contains a 'fusion' peptide at the entrance to a hydrophobic lipid binding cavity (Fig. 1.4). This peptide inserts into lipid membranes, perturbing the orientation of phospholipid thus promoting membrane destabilization. It is thought that the formation of thermodynamically unstable membrane may expose neutral lipids and phospholipids for interaction with the hydrophobic lipid binding cavity of MTP. The extraction of neutral lipids from a membrane is further facilitated by electrostatic interactions between Arg^{781} of MTP and the negative charges on the phosphate groups of bilayer phospholipid molecules. Such interactions would be expected to increase the affinity of the cavity entrance of MTP for lipid.

ApoB was also proposed to interact with intracellular membranes (Spring *et al.*, 1992). In this most cited model of lipoprotein assembly (presented in Fig. 1.6), the nascent apoB polypeptide chain intercalates into the inner leaflet of the ER membrane as part of a process that nucleates intramembrane TG

accumulation. After apoB chain translocation across the ER is accomplished, the TG droplet surrounded by membrane-derived phospholipids, buds into the ER lumen as a soluble nascent lipoprotein particle and enters the secretory pathway. Whether this interaction, mediated by α_1 domain of apoB, occurs at the ER membrane or by means of MTP-dependent transfer of lipid to lumenal apoB remains to be elucidated. There is some contention with this model. The lipid composition of apoB-containing lipoprotein may differ from the lipid composition of lumenal surface of the ER membrane. The phospholipid content of VLDL isolated from rat liver was shown to be 75.5% of PC and 2.6% of PE (Vance & Vance, 1985). In contrast, PE in the rough or smooth microsomes is approximately 25% of the total phospholipids (Bollen & Higgins, 1980). Because MTP does not discriminate among the various phospholipids it transfers, the phospholipid composition of newly synthesized VLDL should be comparable to that of ER.

1.5 Establishment of the requirement of MTP in the assembly of apoB-containing lipoproteins

In 1992, Wetterau et al. published a paper that established the link between MTP and apoB-containing lipoproteins. In intestinal biopsies from an ABL patient, the large 97 kDa subunit of MTP was not detected, and intracellular TG accumulated without the formation of apoB-containing lipoprotein particles (Wetterau *et al.*, 1992). Many studies suggest that MTP is limiting in the

secretion of apoB-containing lipoproteins. Most of ABL patients, who are homozygous for the defective MTP allele, show undetectable apoB plasma levels after a fat load. In another study, using an irreversible, photoaffinity labeled MTP inhibitor in HepG2 cells (human liver carcinoma cell line), MTP activity was decreased and resulted in a proportional decrease in apoB100 secretion (Jamil *et al.*, 1998). Similar experiments have demonstrated that decreased MTP activity correlates with decreased apoB100 and apoB48 secretion in primary rat hepatocytes (Hebbachi *et al.*, 1999) and McArdle RH-7777 cells (rat liver carcinoma cell line) (Jamil *et al.*, 1998). Furthermore, adenoviral–mediated overexpression of MTP in HepG2 cells resulted in a two- to three-fold increase in apoB secretion (Liao *et al.*, 1999). In addition, two independent labs constructed a conditional liver MTP "knockout (KO)" mouse and observed that disruption of MTP gene caused a complete loss of plasma apoB100. ApoB48 was either barely detectable (Chang *et al.*, 1999) or decreased by about 20% (Raabe *et al.*, 1999). The heterozygous $MTP^{+/-}$ mice also showed a somewhat surprising phenotype where MTP activity and mRNA levels were reduced by 50% in both liver and intestine. This phenotype differs from what was found in human heterozygote where the activity and mRNA of MTP is normal (Wetterau *et al.*, 1997). This evidence suggests that in humans, either MTP is not rate-limiting for lipoprotein production, or there is some compensating regulation of MTP in ABL subjects. Noteably, only 18 ABL patients have been studied at the molecular level, and small amounts of circulating plasma apoB100 have been detected in six patients (Aguie *et al.*, 1995), (Glickman *et al.*, 1991), (Herbert *et al.*, 1985). There was

also a low amount of N-terminal degradation fragments found in plasma (Du *et al.*, 1996). ApoB found in the plasma of these ABL subjects is associated only with lipid-poor lipoproteins (Menzel *et al.*, 1990).

1.6 The assembly of VLDL, a general model

There is an extensive controversy regarding the details of VLDL assembly and the exact role of MTP in this process. In one model (Fig. 1.7), as apoB is being synthesized it is translocated across the ER membrane, into the lumen. MTP, which is a lumenal resident, adds lipid cotranslationally to the apoB growing chain. If the lipids are not available, apoB intercalates into the ER membrane on the lumenal side and awaits the lipids. If the pause is too long apoB enters the retrograde transport to the cytosol where it is degraded by the proteosome. As soon as the lipids become available, MTP transfers lipids to apoB post-translationally (after apoB is synthesized). When apoB associates with enough lipids it enters the secretory pathway. Some data suggest that this process occurs in two distinct steps. In the first step, small dense apoB-containing lipoprotein particles are made and enter the ER lumen. In the second discrete step, lipid (primary TG) is added to the particle either by fusion with a preformed lipid droplet that exists in the ER lument or the addition of individual lipid molecules.

Segrest et al., using a computer program called LOCATE, assigned lipid-associating domains in apoB100 and created a lipid pocket model for self-asssembly of apoB-containing lipoprotein particles (Fig. 1.8) (Segrest *et al.*,

1999). In this model, as apoB is being synthesized, the N-terminal domain, namely amphipathic β strands located on the α_1 domain of apoB, alone or more likely as a complex with MTP, forms an intermediate containing a "lipid pocket". The pocket expands through the addition of amphipathic β-strands of apoB and results in the formation of a progressively larger spheroidal VLDL particle.

Another group suggested that the sequences in the C-terminus of apoB29 bind phospholipids and diacylglycerol, whereas sequences between apoB29 and apoB32.5 bind TG, and sequences between apoB 32.5 and apoB 41 account for the marked incorporation of TG at a rate of ~1 TG per 2 amino acids (Carraway *et al.*, 2000). These data imply that β-sheets beyond apoB 29 not only incorporate more lipid but also preferentially recruit the core lipid TG. It has been predicted that the region between apoB21-41 (containing 41 amphipathic β-strands) has a strong lipid binding capacity.

1.7 Evidence supporting a requirement of MTP in the early stages of VLDL assembly

Many groups have observed that MTP plays an important role in the early stages of VLDL assembly. Some studies done on ABL patients show an absence of apoB-associated lipoproteins of any density (lipoproteins can be secreted as poorly-lipidated particles) (Wetterau et al., 1992). It has been suggested that the block of the assembly of VLDL that takes place in MTP-deficient cells must occur before the particle is formed. Studies performed on HeLa (Gordon *et al.*,

1994) and COS (Leiper *et al.*, 1994) cell lines (non-hepatic cell lines) support the same idea. These cells do not make lipoprotein particles; however, when the cells are transfected with cDNA encoding apoB and MTP, small dense particles are assembled. When these cells are transfected with apoB cDNA alone, no particles are formed.

There are two suggested sites on apoB were MTP binds: one is the first 300 residues of β-barrels of apoB and MTP, and second at residues 512-721 of apoB and residues 517-603 of MTP (Bradbury *et al.*, 1999), (Nicodeme *et al.*, 1999). In this studies apoB subfragments were used to map the interaction of apoB with MTP in a yeast two-hybrid system. The exact location of the binding sites is still being disputed. Co-immunoprecipiation studies show a physical interaction between apoB and MTP at the early stages of VLDL formation which decreases as the assembly process proceeds (Wu *et al.*, 1996). It has been also implied that while the proper folding of the globular N-terminal and extensive modification of apoB is independent of MTP, however, apoB proper conformation serves as a prerequisite for the binding of MTP to apoB (Gretch *et al.*, 1996). Therefore, the binding of MTP to apoB may be initiated shortly after the N-terminal is folded. Another study done by Grand-Perret supporting this belief indicated that the initiation of apoB100 translation was not affected by MTP inhibition (Benoist & Grand-Perret, 1997) .

A relationship between the length of apoB and the requirement of MTP for VLDL secretion has also been established. The short forms of apoB (apoB15 to apoB23) are secreted in a MTP-independent manner (Nicodeme *et al.*, 1999).

Fragments longer than apoB23 but shorter than apoB51, are also secreted in the absence of MTP, however, the amount of these apoB-containing lipoproteins is decreased and in a lipid-poor status. This suggests that the MTP packaging of apoB with lipids is a crucial step and the acquired lipids help apoB to assume a proper conformation required for successful secretion.

1.8 Evidence supporting a requirement of MTP in the later stages of VLDL assembly

Alexander et al. identified apoB-free lipid droplets in the smooth ER using electron microscopy (Alexander *et al.*, 1976). They proposed that lipid droplet fuses with apoB-containing particle at the junction of the smooth and rough ER to form a lipid-rich particle.

Recent studies done by Raabe et al. in MTP KO mice (Raabe *et al.*, 1999) observed secreted apoB48, which was poorly lipidated and also lipid accumulation in the cytosol. These studies suggested that MTP is involved in the mobilization of TG across the ER membrane, and that the absence of MTP prevents the formation of the lumenal TG droplet that fuses with the nascent apoB particle.

1.9 Regulation of VLDL assembly and secretion by the availability of lipids

The secretion of apoB is dependent on lipids and the supply of TG, phospholipids cholesterol and cholesteryl esters probably determines how much of apoB-containing lipoproteins are secreted. When lipids are not available, apoB secretion is decreased and degradation is enhanced. The phospholipids and cholesterol utilized for the formation of the VLDL coat are synthesized on ER membranes. TG used for the core of VLDL comes mostly from a cytosolic droplet and some TG from *de novo* synthesis. PC is the major phospholipid, comprising of 80% of the total phospholipid content of VLDL. PC deficiency causes a 60% reduction of VLDL in a mouse model, and relative enrichment in PE (Yao & Vance, 1989). A decrease in PC biosynthesis causes incorrect assembly of nascent VLDL and subsequent degradation by a quality control protease in a post-ER compartment (Fast & Vance, 1995) which is distinct from degradation of apoB in the ER.

TG is synthesized in the liver on the cytoslic side of the smooth ER and it is transferred to the cytosolic lipid droplet or to the lumen of ER, the assembly site of VLDL (Gibbons *et al.*, 2000). The suggested steps are summarized in Fig. 1.9. The cytosolic lipid droplet TG contributes about 70% of VLDL TG (Gibbons & Burnham, 1991) and in order to be incorporated into VLDL, cytosolic TG has to be hydrolyzed by lipolysis.

Lehner et al. presented evidence for the involvement of an enzyme called triacylglycerol hydrolase (TGH) in the assembly of VLDL (Lehner *et al.*, 1999). The expression level of TGH correlates with the changing pattern of VLDL

secretion during neonatal development. TGH appears to be in close contact with lipid storage droplet. The products of lipolysis, DG, MG, and FFA are more stable and soluble within the phospholipid membrane environment than is TG and these products are then re-esterified to TG. The sites of TG synthesis from extracellular fatty acids, and from lipolytically-derived fatty acids, are physically distinct (Gibbons *et al.*,2000). Unfortunately, little is understood of what actually occurs between theses events.

Furthermore, it has been demonstrated that fatty acids stimulate TG synthesis. For example, in primary cultured hepatocytes, oleic acid induces VLDL secretion (Dashti & Wolfbauer, 1987). The ability of fatty acids to stimulate apoB secretion is dependent on the type of the fatty acid. The reduction is observed with the increasing chain length and degree of saturation (18:1>14:0>16:0>18:0) (ref). In addition, fish-oil derived fatty acids, such as eicosapentaenoic and docosahexaenoic acids, have been shown to decrease apoB and TG secretion in comparison to other FA (Wong *et al.*, 1989).

The requirements of cholesterol and cholesteryl ester in VLDL assembly and secretion are still uncertain and under intense investigation. Both types of lipids are found within VLDL, however, there is no evidence to show that they act as specific regulators of VLDL secretion. The rate-limiting enzyme of cholesterol synthesis, HMG-CoA reductase, is inhibited by well-characterized statin drugs, which decrease VLDL assembly, may contribute to the decline of plasma cholesterol and cholesteryl ester, and consequently reduce the risk of heart disease (Khan *et al.*, 1990). Therefore, the restricted availability of cholesterol and

cholesteryl ester can be a rate-limiting factor in the assembly of VLDL. In a study performed by Dashti et al, addition of 25-hydroxycholesterol or LDL to the medium of HepG2 cells increased apoB-containing lipoproteins secretion which corresponded to elevation of cholesteryl ester mass (Dashti *et al.*, 1992).

1.10 Degradation of ApoB

A significant control over the levels of secreted apoB-containing lipoproteins can be achieved through co- and post-translational degradation. It has been recognized that misfolded apoB moves from ER lumen into the cytosol for degradation as a result of decreased MTP activity (Yao *et al.*, 1997) or the unavailability of lipids (Dixon & Ginsberg, 1993). This degradation process occurs via the cytosolic ubiquitin-proteosome where a ubiquitin-conjugating enzyme catalyzes the covalent attachment of ubiquitin to apoB followed by the degradation by proteosome. Degradation has been shown to be reduced by inhibitors such as lactacystin. However, misfolded apoB does not accumulate in the cytosol (Zhou *et al.*, 1998) but remains associated with the ER (Mitchell *et al.*, 1998). There are additional proteolytic pathways that may degrade apoB. The finding of N-terminal apoB peptides in the rough microsomes obtained from primary rat hepatocytes suggested that apoB could be degraded in the same compartment it is synthesized (Davis *et al.*, 1989). Fully translocated apoB can also be degraded in the ER lumen by process involving ER-60 (Adeli *et al.*, 1997) or DTT inhibitable (Wu *et al.*, 1997) proteases. Furthermore, apoB that resides in

the lysosomal/endosomal compartment has been proposed to be degraded by chloroquine-inhibitable protease (Wang *et al.*, 1995). Another mechanism that is responsible for degradatation of apoB involves a re-uptake pathway. After a fully assembled apoB-containing lipoprotein has been exported outside of the cell but before it diffuses away from the cell, the particle may bind to the LDL receptor (Williams *et al.*, 1990). LDL receptors are located on the cell surface and together with heparan sulfate proteoglycans bring the nascent particles back into the cell to lysosomes where the degradation follows.

1.11 ApoB-containing lipoproteins in post-ER compartments

The synthesis of apoB takes 8-15 min (Bostrom *et al.*, 1986). Shortly after that, apoB can be found in the lumen of ER. The nascent lipoproteins are first detected by electron microscopy in the smooth ER. Lipoproteins are held within the ER for over a 30 min period before entering the Golgi apparatus (Bostrom *et al.*, 1986). This delay may be attributed to the slow process of lipid addition and to apoB acquiring the proper conformation that allows the lipoprotein to proceed out of the ER. In Golgi, a further addition or exchange of phospholipids and neutral lipids may occur (Bamberger & Lane, 1988) or the particle can be degraded by DTT inhibitable proteases (Wu *et al.*, 1997). Moreover, in the Golgi compartment, apoB undergoes modifications such as hydrolysis of mannose side chain and O-linked glycosylation (Vauhkonen *et al.*, 1985). Evidence suggests that only a fraction of total synthesized apoB actually enters the secretory

pathway and is later found in the medium (Borchardt & Davis, 1987). The degree of degradation differs between cell lines.

1.12 Thesis objective

The focus of this study is the mechanisms of action of MTP and the role it plays in the assembly and secretion of apoB-containing lipoproteins. From a variety of approaches, we accumulated data that suggests MTP requirement in the mobilization of TG from cytosolic lipid stores and delivery to either a growing apoB chain or lumenal lipid droplet.

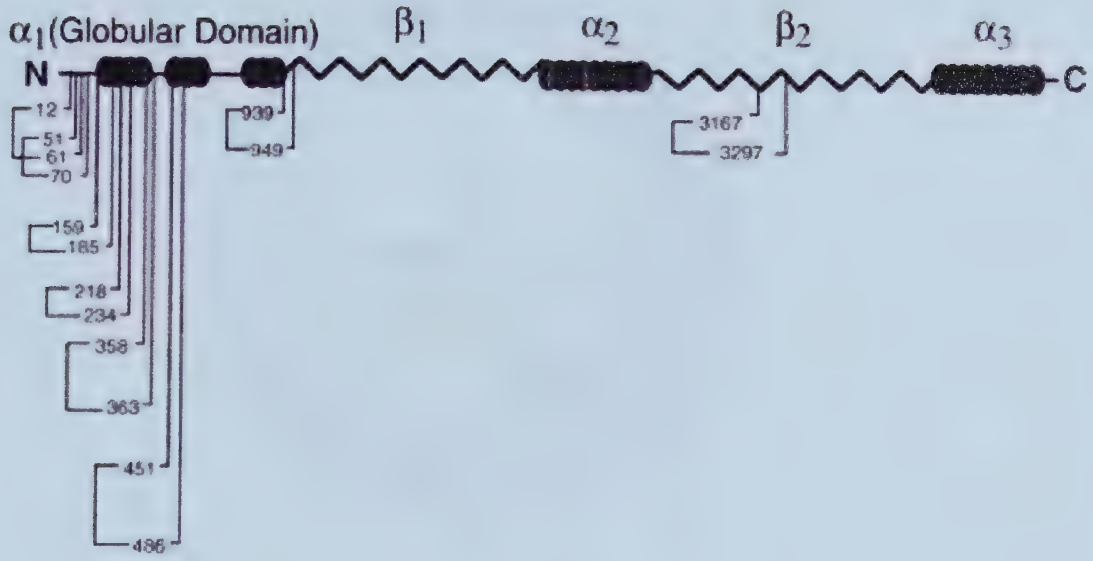


Fig. 1.1. **Domain structure of apoB**. ApoB has been described in terms of a pentapartite structure in which three amphipathic α-helical domains (α1, α2 and α3) alternate with two domains predicted to form amphipathic ß-sheet structures (ß1 and ß2). Bracketed numbers indicate the position of cysteine residues involved in disulfide bond formation. Fig. adapted from Shelness *et al.*, 1999.

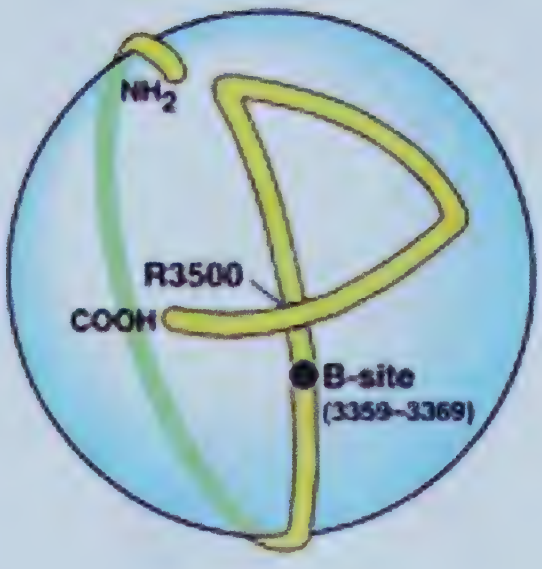


Fig. 1.2. **Schematic of apoB associated with LDL.** The first 89% of apo-B100 enwraps the LDL particle like a belt, and the carboxyl-terminal 11% constitutes a bow that crosses over the belt, bringing the carboxyl-terminal portion of apo-B100 close to amino acid 3500. ApoB100 binds to the LDL receptor via Site B (residues 3359-3369) and the bow serves as a modulator element that can alter the affinity of receptor-binding domain of apo-B100. Fig. adapted from Boren *et al.*,1998.

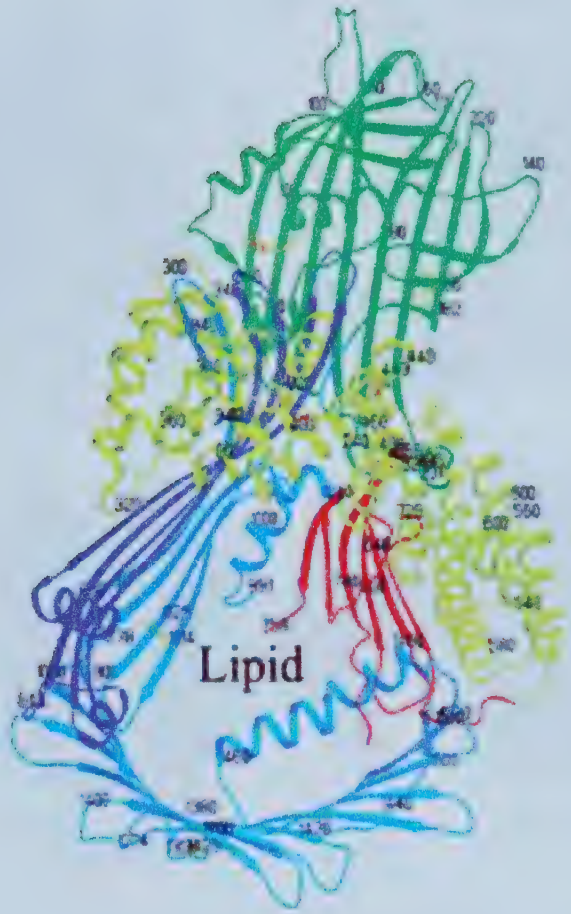


Fig. 1.3. **Ribbon diagram of the LV.** The N-terminal β-barrel is shown in green, the helical domain in yellow, and the C-sheet domain in red. β-Strands 2-5 and helices A and B of the A-sheet are shown in dark blue. Strands 6-23 are in cyan. Fig. adapted from Read *et al.*, 2000.

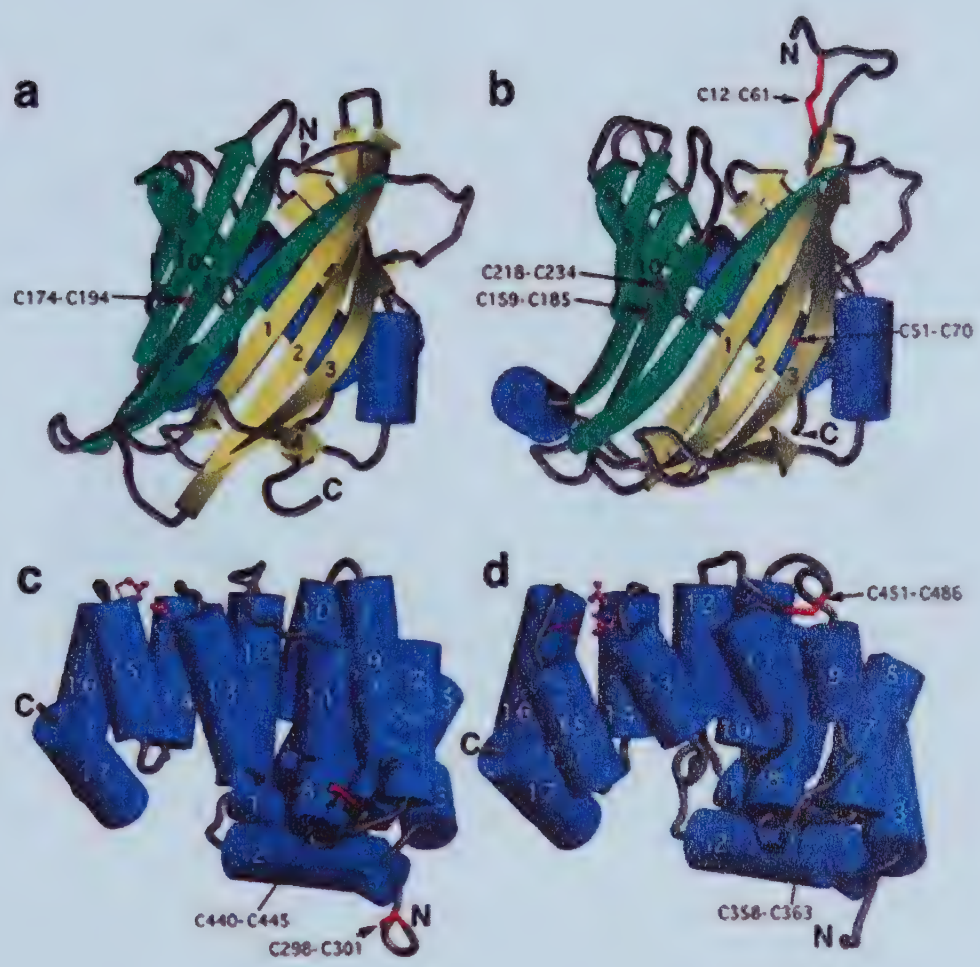


Fig. 1.4 **Molecular models of MTP and apoB based on the atomic coordinates of LV**. a, The amino-terminal β-sheet of MTP. Of the 13 β-strands, 11 are arranged in a barrel-like conformation. Strands 7-12 correspond to the homodimerization interface of lamprey LV and are depicted in green. b, The amino-terminal β-barrel of apoB. c, The predicted α-helical domain of MTP comprised of helices 1-17, arranged in inner (even numbered) and outer (odd numbered) layers. d, The α-helical domain of apoB comprised of helices 1 to 17. Fig. adapted from Mann *et al.* 1999.

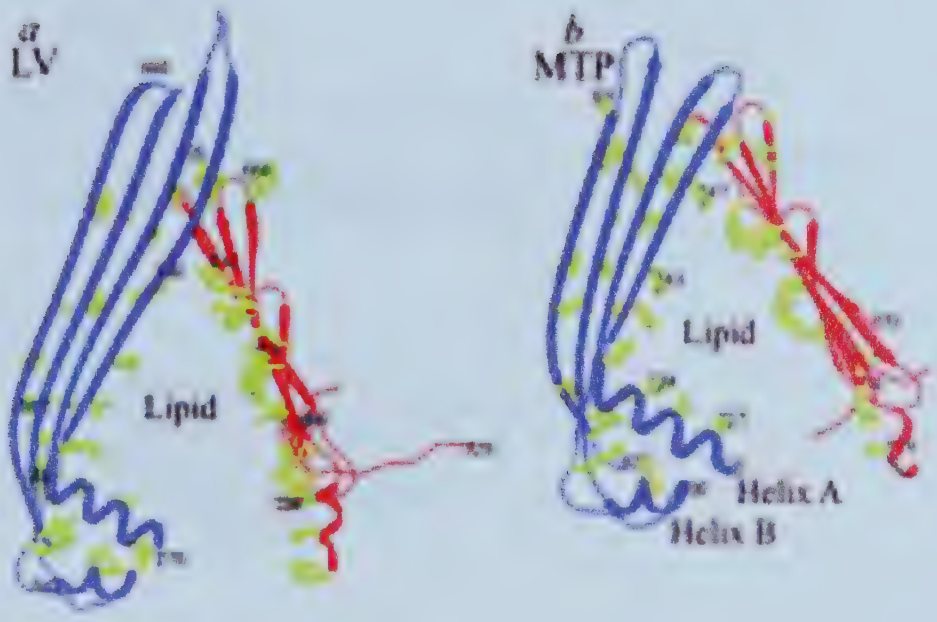


Fig. 1.5. **Ribbon diagrams for the lipid binding cavities of LV and MTP**. a, expanded view of β-strands 2-7 of the C-sheet of lamprey LV (red) and of β-strands 2-5 and helices A and B of the A-sheet (blue). b, molecular model of the lipid binding cavity of MTP based on the atomic coordinates of lamprey LV. Fig. adapted from Read *et al.,* 2000

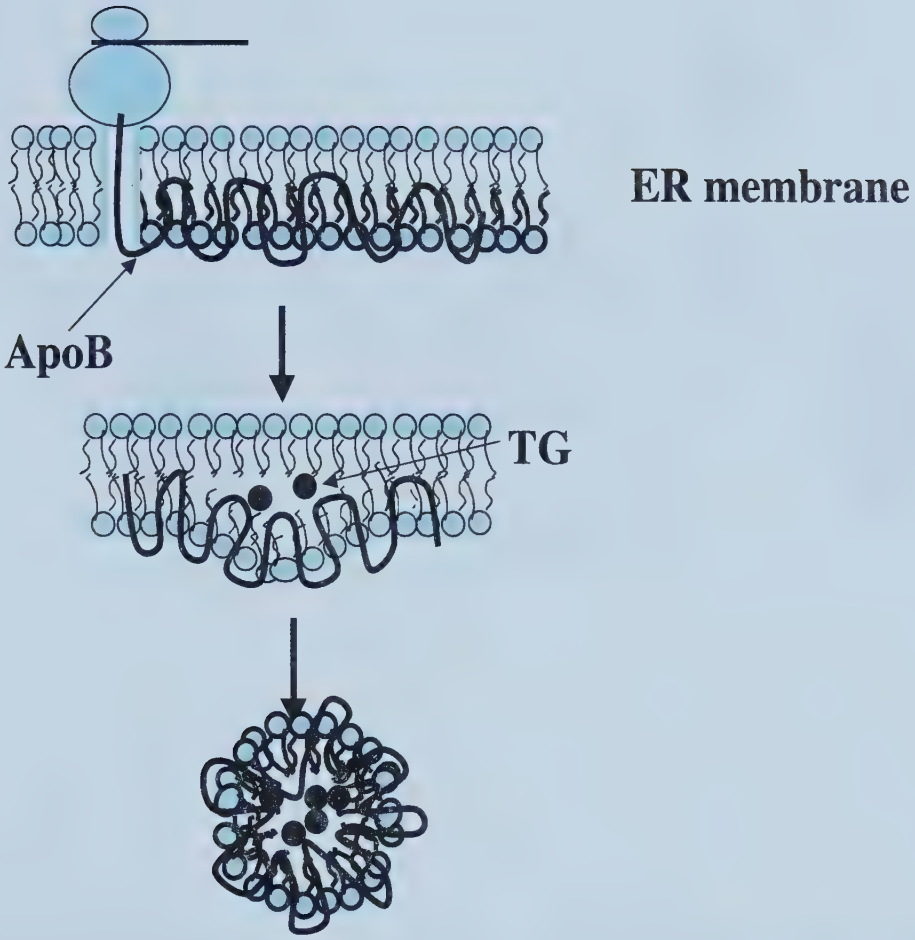


Fig. 1. 6. **Hypothetical function of the α_1 domain of apoB**. As apoB is being synthesized, the α_1 domain of apoB may associate with the inner leaflet of the ER membrane followed by the mobilization of cytosolic TG. At some point during the packaging of TG with apoB, the particle may bud from the membrane into the lumen of ER.

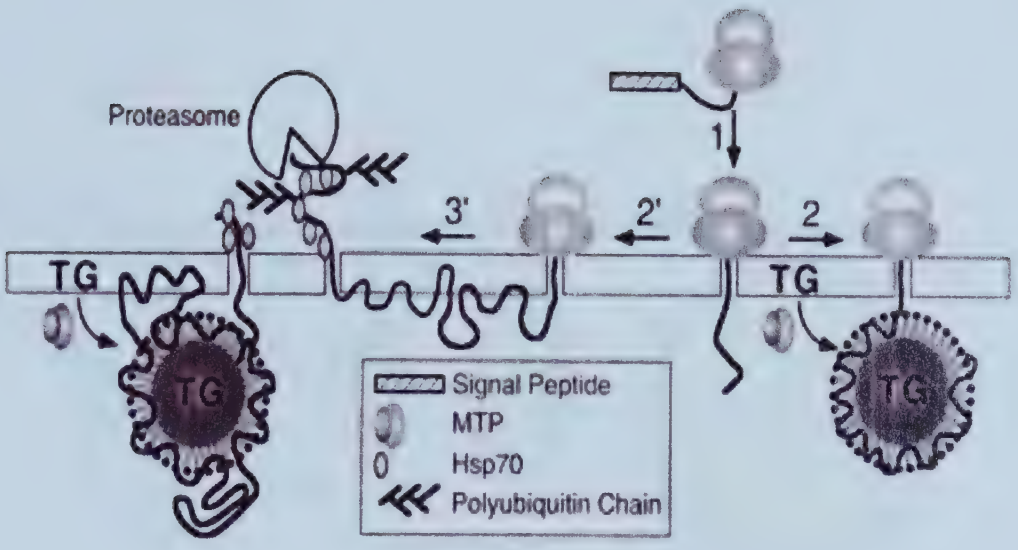


Figure 1.7. **The assembly and degradation of apoB containing lipoproteins.** ApoB undergoes signal peptide-mediated targeting to the ER membrane (Step 1). In the presence of MTP and "secretion-coupled" lipid, apoB initiates cotranslational lipoprotein assembly (Step 2). Apolipoprotein B that fails to assemble with lipid (Step 2') may aggregate or insert into the lipid bilayer and is targeted for retrograde translocation and proteasome-mediated degradation (Step 3'). This degradation pathway may occur both cotranslationally or post-translationally. These fully translocated and misassembled forms of apoB are presumably the substrates for the post-translational assembly of membrane-bound apoB that can be induced by stimulation of triglyceride synthesis and mobilization. Fig. adapted from Shelness *et al.,* 1998.

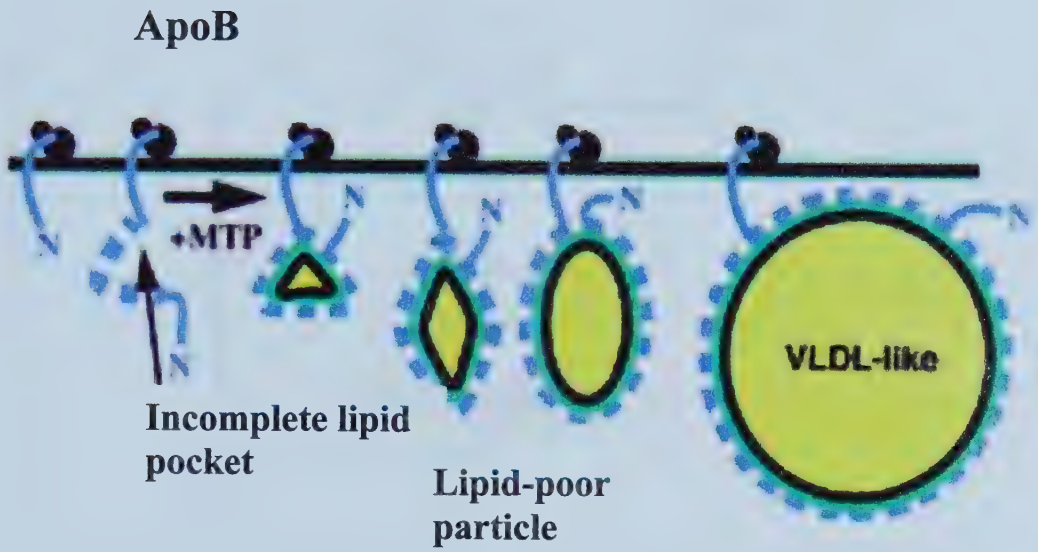


Fig. 1.8. **"Lipid pocket" model for assembly of apoB-containing lipoprotein particles**. First, an incomplete "lipid pocket" is formed by amphipathic ß strands (blue dashes) located in the α_1 domain of apoB. MTP is required for this pocket to fill with lipid (yellow, neutral lipid; green, phospholipid head groups; black, fatty acyl chains), perhaps acting as a co-structural element to complete the pocket. It has been hypothesized that the α helical portion of MTP that lines the lipid pocket (Fig. 1.5b) represents the domain of MTP that binds to the positively charged N-terminal portion of the α domain of apoB (Fig. 1.4d). Once the pocket is filled, additional amphipathic ß strands from the ß1 domain of apoB are co-translationally added, allowing the "lipid pocket" to expand until defined lipoprotein particles of lipid-poor, then lipid-rich VLDL density result by addition of individual lipid molecules or by the fusion with a lipid droplet. A figure adapted from Segrest *et al.*, 1999.

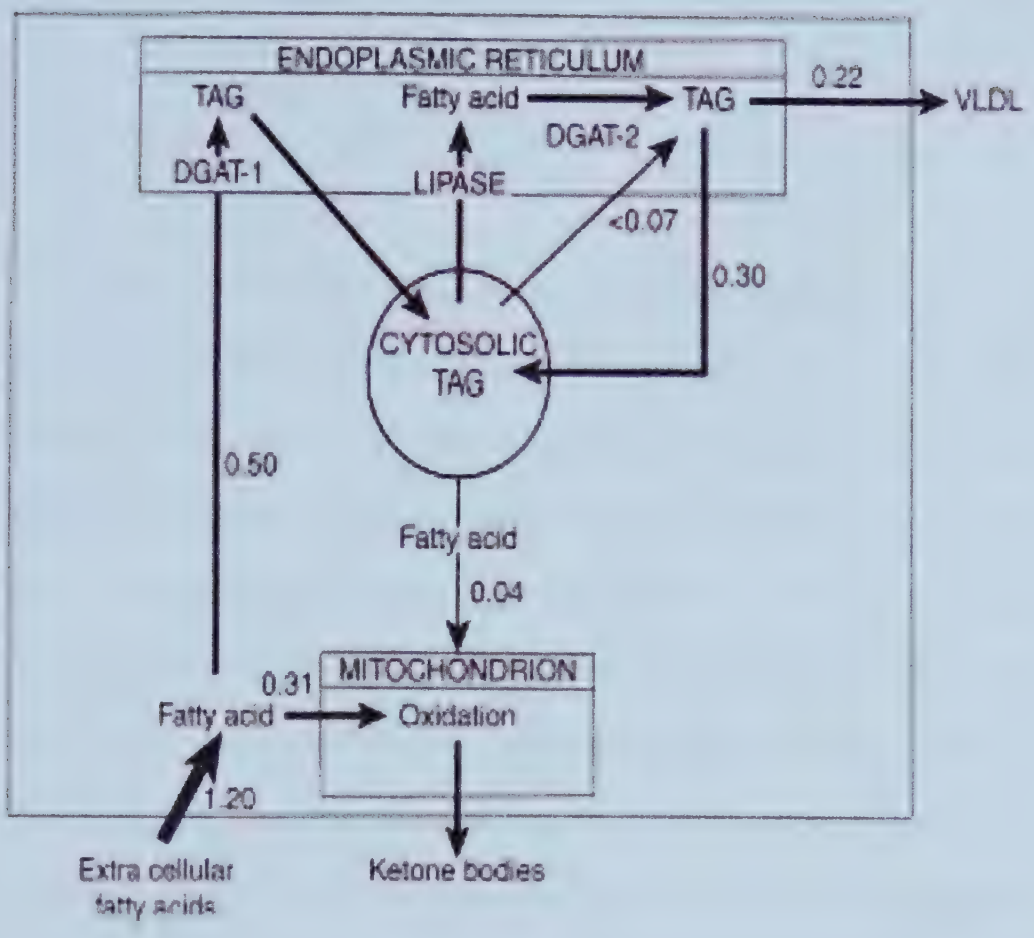


Fig. 1.9. **Pathways of FA and TG utilisation for hepatic storage, oxidation and secretion as VLDL**. Extracellular fatty acids entering the cell are esterified in the ER to TG, which is transferred to a cytosolic storage pool. TG required for VLDL synthesis is recruited from this pool by a process of lipolysis to give FA, MG and DG. These lipolysis products are re-esterifed to TG. The sites of TG synthesis from extracellular fatty acids, and from lipolytically derived FA, are physically distinct. Fig. adapted from Gibbons et al., 2000.

Chapter 1 - Introduction

1.1 Introduction

Heart disease is the major cause of death in Western countries. The primary cause is the deposition of Low Density Lipoprotein (LDL)-derived cholesterol on the arterial walls. These lipoprotein particles arise from the initial assembly of Very Low Density Lipoproteins (VLDL) in the liver. Subsequently, VLDL is secreted into the plasma and a complex series of enzymatic reactions converts VLDL to LDL. The primary function of the lipoproteins is to deliver lipids such as triacylglycerol (TG), cholesterol and cholesterol ester to the peripheral tissues. The lipid composition of the lipoprotein depends on the diet of the individual and the proper function of all enzymes, receptors and ligands that determine the lipoprotein metabolism. Great effort has been invested into understanding the molecular mechanisms that control assembly and secretion of lipoproteins. The accomplishments have already assisted in the development of effective therapeutic agents, which lower plasma LDL and ultimately reduce the mortality associated with heart disease.

The major requirements for VLDL assembly were established by studying genetic disorders that manifest as a decrease or elimination of plasma VLDL. Hypoabetalipoproteinemia is a genetic disorder that is characterized by the absence of apoB-containing lipoproteins from the plasma (Aguie *et al.*,1995). Studies with patients having this disorder led to the discovery of a major protein

Chapter 2 - Experimental Procedures

Materials

Dulbecco' Modified Eagle Medium (DMEM), Hank's EGTA solution, Hank's collagenase solution, penicillin-streptomycin, and fetal bovine serum were obtained from GibcoBRL, Burlington. Minimum essential medium Eagle (methionine-free), fatty acid-free bovine serum albumin, oleic acid, phenylmethylsulfonyl fluoride, leupeptin, N-Acetyl-Leu-Leu-norleucinal (ALLN), trypsin, trypsin inhibitor, digitonin, collagenase type I, insulin, and tunicamycin were purchased from Sigma, Oakville Ont. [^{35}S]methionine, [^{3}H]oleic acid, Protein A Sepharose CL-4B, Amplify, and rainbow protein molecular weight markers were from Amersham Pharmacia. Anti-apolipoprotein B human antibody was purchased from Boehringer Mannhein (Roche), Laval, Quebec. Collagen coated cell culture dishes were obtained from BIOCOAT (VWR), Mississauga Ont. TLC plates were from Merk and all chemicals used for SDS-PAGE were from Bio-Rad. MTP inhibitor BMS-197636 was obtained from Bristol Myers Squibb, New Jersey, and dissolved in DMSO. C57BL/6 mice were purchased from Jackson Laboratory.

Methods

Preparation and culture of mouse hepatocytes

Adult male mice fed regular chow diet were sedated and anesthetized by IP injection of somnotol (0.022 ml/50 g body weight) before surgical incision and perfusion through the portal vein. First, the Hank's EGTA solution was run through until the liver was clear of blood followed by Hank's collagenase solution (50 units/ml) until liver became soft. Digested liver was homogenized and washed three times with DMEM. The isolated hepatocytes were plated in DMEM containing antibiotics and 10% FBS onto a collagen-coated dishes (2 million cells per dish). The cell number and viability was determined using trypen blue exclusion (dead cells absorb the die and turn blue). Following cell attachment, which usually takes 2 to 3 hr, fresh DMEM media with antibiotics and FBS was added and cells were left overnight. The next morning, the media were changed to FBS free with or without oleic acid (0.325 mM bound to BSA) and pulse-chase experiments were performed where either protein or lipids were labeled.

Metabolic labeling of protein and lipids

Translocation study - Cells incubated overnight with DMEM containing FBS were washed twice with methionine-free media before the addition of

methionine-free media containing 0.325 mM of oleic acid +/- MTP inhibitor for 2h. 100 μCi/ml of [^{35}S]methionine was added for 15 or 30 min. The labeled cells were washed with

CSK buffer (0.3 M sucrose, 0.1 M KCl, 2.5 m $MgCl_2$, 1 mM sodium-free EDTA, 10 mM PIPES, pH 6.8) and then incubated in digitonin (75 μg/ml) in CSK buffer for 5 min on ice. CSK washed and digitonin-treated cells were subjected to trypsin (100 μg/ml) digestion for 10 min. The hydrolysis was stopped with the addition of trypsin inhibitor (1 mg/ml), PMSF (1 mM), leupeptin (0.1mM). The digitonin concentration and time it takes to permeabilized cell membrane was studied by using a lactate dehydrogenase assay, a cytosolic enzyme whose product is released upon permeabilization. The trypsin sensitivity was determined with phosphatidylethanolamine N-methyltransferase (PEMT) and cholinephosphotransferase (EPT) assays. Enzymes that are microsomal membrane bound, lose their activity with trypsin treatment. Half of the dishes of the experiment were collected in a solubilization buffer (0.05M Tris-HCl, 0.15M NaCl, 0.005M EDTA, 0.001M PMSF, 1% Triton-X100, pH 7.4) and subjected to immunoprecipitation of total apoB (this includes apoB associated with inner ER membrane and apoB located in the ER lumen) and the other half was homogenized in sucrose with Dounce homogenizer and subjected to carbonate extraction and sucrose gradient ultracentrifugation of lipoproteins. The lumenal lipoproteins were separated into 12 fractions of different densities (d=1.21-1.006 g/ml) and apoB immunoprecipitated from each fraction, analyzed by SDS-PAGE and fluorography and quantified with densitometric scanning.

The amount of total lumenal lipids and the lipid content of lipoproteins of different buoyancy separated by ultracentrifugation was also determined. Cells incubated overnight with DMEM containing 10 % FBS were washed twice with DMEM FBS-free media followed by the addition of DMEM containing +/- MTP inhibitor. After 2 h, cells were pulsed with 10 μCi/ml of [^{3}H]oleic acid for 30 min and then subjected to carbonate extraction. Total TG of isolated microsomes and the TG content of lumenal lipoproteins were resolved by thin layer chromatography (TLC) with heptane/isopropyl ether/acetic acid; 60:40:4. Plates were visualized by iodine, bands scraped and radioactivity determined by scintillation counting. To get high enough radioactivity counts of TG isolated from lipoproteins two dishes had to be combined.

Isolation of lumenal TG

Cells incubated overnight with DMEM containing 10 % FBS were washed twice with DMEM FBS-free media followed by the addition of DMEM containing +/- MTP inhibitor. After 2 h, oleate deprived cells were pulsed with 10 μCi/ml of [^{3}H]oleic acid for 30 min with the excess of fatty acids. Cells were homogenized and microsomes recovered. The isolated microsomes were treated with 0.5 M KCl to remove attached cytosolic lipid droplet and separated into lumenal contents and membrane fractions with a sodium carbonate method described below. ApoB was immunoprecipitated from the lumenal contents and

the remainings used to determine the amount of TG by lipid extraction and TLC analysis.

Secretion study

Determination of the amount of secreted protein - Hepatocytes were washed and preincubated with methionine-free media containing 0.325 mM oleic acid, +/- MTP inhibitor for 2 h followed by the addition of 100 μCi/ml [^{35}S]methionine for 4 hr. The media was collected and half of the samples were used to immunoprecipitate total amount of secreted apoB and the other half was ultracentrifuged to separate lipoproteins of different densities and then immunoprecipitated for apoB. As before, apoB was resolved on SDS-PAGE, visualized by fluorography and quantified with densitometric scanning.

Determination of the amount of secreted lipids - Cells were washed and incubated with FBS-free DMEM for 2 hr, +/- MTP inhibitor and then pulsed with 5 μCi/ ml of [^{3}H]oleic acid for 4 hr, +/- MTP inhibitor. Next, the media was collected and TG in the total, as well as in each density fractionated sample was studied.

Carbonate extraction

The isolated microsomes were treated with 1M Na_2CO_3 to separate microsomal membrane from lumenal contents. After 25 min incubation at room

temperature, 25 mg/ml of bovine serum albumin (BSA) was added to the sample to bind free lipids followed by an ultracentrifugation at 35,000 rpm for 90 min at 21°C in Beckman SW60 rotor. The supernatant containing lumenal contents was taken away from the pelleted membranes and pH adjusted back to 7.4 by addition of 60 µl/ml of 10% acetic acid. The obtained samples were placed on a sucrose gradient for density fractionation. Membranes, on the other hand, were resuspended with PBS for immunoprecipitation. All the solutions contained protease inhibitors.

Sucrose gradient ultracentrifugation of lipoproteins

Lipoproteins present in the microsomal lumen or media were resolved according to their densities by sucrose gradient ultracentirfugation. The samples in 12.5% sucrose were placed on 49% and 20 % of sucrose and topped with PBS (all solution contained protease inhibitors). The layered samples were ultracentrifuged in Beckman SW40 rotor at 35,000 rpm for 60 h at 12 °C (Rustaeus *et al.*, 1995). The ultacentrifugation provided us with 12 fractions of densities ranging from 1.21-1.006 g/ml. Bottom fractions (1 to 6) represent lipid-poor particles top fractions represent lipid-richer particles.

Chapter 3 – Results

3.1 Inhibition of TG secretion with an MTP inhibitor, BMS-197636.

MTP plays an important role in the assembly of apoB-containing lipoproteins. However, the exact mechanisms of action of this protein have not been determined and the question addressed in this study is at what stage of lipoprotein assembly is MTP required. To study the requirements of MTP we used an inhibitor, called BMS-197636 (MTPI), obtained from Bristol-Myers Squibb, on mouse primary hepatocytes. This compound was shown to be highly effective in inhibiting human MTP TG transfer activity *in vitro* at subnanomolar potency (IC_{50}=36 nM) (Wetterau *et al.*, 1998). The decrease in apoB secretion in HepG2 cell line occurs within10 min of incubation in the presence of this drug at very low concentrations (ED_{50}=3 nM) (Wetterau *et al.*, 1998).

To find the effective concentration of MTPI, we examined the secretion of TG from primary mouse hepatocytes with different concentrations of the inhibitor. Hepatocytes were preincubated for 2 hours with increasing concentrations of MTPI ranging from 0 to 10 μM in lipid-free media. [^{3}H]oleate was then added to the media for an additional 4 hr to monitor TG secretion. In addition, the media were supplemented with 0.375 mM of non-labeled oleate; a presence of this concentration of oleate had previously been shown to induce the assembly and secretion of apoB-containing lipoproteins (White *et al.*, 1992). The

media were then collected, analyzed for the presence of labeled TG by lipid extraction, followed by thin layer chromatography. The results are shown in figure 3.1. Inhibiton of TG secretion was found to be proportional to the concentration of MTPI. The concentration that resulted in a ~50% reduction in TG secretion was found to be 1μM. Administration of 10 μM of MTPI resulted in an approximate 80% reduction of TG secretion. These concentrations are higher than found in HepG2 cells. Perhaps BMS-197636 is not readily absorbed by mouse primary hepatocytes and therefore, concentrations greater than sub-nanomolar are required. Thus, we found that 10μM MTPI would provide sufficient inhibition of TG secretion. It should be mentioned here that the inhibitory effect persisted for only 4 hr. Beyond this time, sustained inhibition could only be achieved if MTPI was re-administered (not shown). This experiment provided a means of measuring the MTPI's inhibitory potential with regards to TG secretion from primary hepatocytes and was carried out routinely to ensure the efficacy of inhibition throughout the course of all experiments.

3.2 The involvement of MTP in the translocation of apoB across the ER membrane

It has been suggested that MTP is not required for the translocation of apoB48 across the ER membrane and into the ER lumen (Rusinol *et al.*, 1997). However, this study was performed in canine pancreatic microsomes, which do not express MTP. To examine further the requirement of MTP in the process of

translocation of apoB48 and apoB100 across the ER membrane, murine primary hepatocytes were labeled with [^{35}S]methionine for 15 or 30 min; apoB100 synthesis occurs in about 10 min and it takes another 30 min before apoB100 is detected as a secretion product (Bostrom et al., 1986). Next, the cells were permeabilized with digitonin then treated with trypsin to digest proteins residing in the cytosolic compartment. Under these conditions, apoB that crossed the ER membrane and entered the lumen of ER or was associated with the inner lumenal membrane should be protected from trypsin digestion (Adeli *et al.*, 1994), (Macri & Adeli, 1997). After 15 min labeling, in cells that had been treated with MTPI both apoB48 and apoB100 were found in the microsomes and were protected from trypsin digestion (Fig. 3.2). However, the amount of apoB100 recovered from this fraction by immunoprecipitation was ~30% less than in to control cells (Fig. 3.2). Hepatocytes that were pulsed-labeled for 30 min in the presence of MTPI displayed a more substantial decrease (about 80%), in the amount of apoB100 protected from trypsin digestion. ApoB48 levels were the same as in control cells. These results suggest that in the presence of MTPI, both apoB100 and apoB48 can cross the ER membrane and possibly insert into the inner membrane.

The decreased apoB100 associated with ER membranes in the presence of MTPI may be due to either inhibition of translocation of apoB100 by MTPI or increased retrograde transport to the cytosol where it allocated to the proteaosome for degradation. To examine the possible involvement of such a retrograde transport to the proteaosome, we treated cells with two different proteaosome

inhibitors. It was shown previously that in HepG2 cells ALLN and MG123 inhibit proteaosomal degradation and cause accumulation of apoB in the lumen but no increase in the secretion is observed (Benoist & Grand-Perret, 1997). Unfortunately, the primary hepatocytes were found to be too sensitive to the proteasome inhibitors (at concentrations suggested by previous studies), which severely compromised cell viability (not shown). Consequently, the levels of apoB in the ER lumen of cells treated with proteosome inhibitors were reduced and the effect was independent of the presence or absence of MTP inhibitor.

3.3 Effect of MTPI on the lipidation of apoB

With some ABL patients apoB was found in the lumen of ER. However, the protein was quickly degraded and no apoB was detected in their plasma (Glickman *et al.*, 1991). To investigate the effect of MTP inhibition on the translocation of apoB into the lumen of the ER and subsequent addition of lipids, we isolated lumenal contents of microsomes isolated from mouse hepatocytes, and fractionated the lumenal apoB-containing lipoproteins according to their density. In this experiment, apoB was labeled with [^{35}S]methionine for 30 min in the presence or absence of MTPI. Microsomes, (which includs ER and Golgi) were subjected to carbonate extraction to disrupt membranes. Membranes and lumen contents were separated by centrifugation. The supernatant, consisting of apoB-containing lipoproteins was placed on a sucrose gradient and ultracentrifuged for 60 hr to separate lipoproteins of densities ranging from 1.21

to 1.006 g/ml. Twelve fractions were collected and apoB was recovered from each by immunoprecipitation. The results presented in Fig. 3.3 demonstrate that in control hepatocytes, apoB100 is found in the microsomal lumen and is associated with lipoproteins of densities from 1.21 to 1.006 g/ml. Fractions 10 and 11, corresponding to lipid-rich particles, contained noticeably more apoB100. The same trend was observed for control apoB48. In hepatocytes treated with MTPI, apoB 100 was not detectable in the lumen (Fig. 3.3). Thus apoB100 may be capable of associating with ER membranes in the presence of MTPI (Fig. 3.2) but does not become lipidated to form lipoprotein particles within the soluble fraction of the lumen. By contrast, apoB48 is capable of becoming lipidated in the presence of MTPI. However, unlike apoB in control cells, the extent of apoB48 lipidation is largely restricted to lipid-poor particle densities (Fig. 3.3). The total amount of apoB48 from all 12 fractions of MTPI treated cells (Fig. 3.3) was the same as that immunoprecipitated from control cells.

Furthermore, we measured the amount of TG in lipoproteins isolated from the lumen of microsomes that accumulated during the 30 min time-course of the experiment. Lipids of the hepatocytes were labeled with [^{3}H]oleate for 30 min, microsomes were isolated, and membranes were separated from the lumenal contents by carbonate extraction and ultracentrifugation as described in the previous experiment. Subsequently, apoB-containing lipoproteins were immunoprecipitated with anti-apoB antibody under non-denaturing conditions (PBS was used), which allows isolation of the entire particle in its nascent form (the apoB association with lipids is intact). Isolated lipoproteins were then

solubilized with a detergent containing TritonX-100, and lipids were extracted and analyzed by TLC. It was found that TG from lumenal apoB-containing lipoproteins of MTPI treated cells was reduced by 40% in comparison to that in control hepatocytes (Fig. 3.4). Additionally, the efficiency of the immunoprecipiation of apoB-containing lipoproteins was addressed. After the standard immunoprecipitation procedure, the remaining lumenal contents were evaluated for the presence of apoB. Western blotting results confirmed the absence of apoB (not shown), which indicates the complete recovery of this protein from the lumen. In the presence of MTPI, lipid delivery to apoB was compromised. Therefore, the MTP inhibition results in a decreased TG delivery to apoB, which becomes only partially lapidated (Fig. 3.3) as in the case of apoB48, which is present mainly on lipid-poor particles, or degradation as observed with apoB100.

3.4 Secretion of apoB48 and apoB100 in the presence of MTPI

Studies performed on primary hepatocytes derived from liver-specific MTP knockout mice by Young's lab revealed that plasma apoB 100 was abolished, while apoB48 plasma levels were virtually unchanged (Raabe *et al.*, 1999). In contrast, Chan's MTP liver-specific knockout mice displayed a decreases in both plasma apoB100 and apoB48 (Chang *et al.*, 1999). We therefore, investigated the influence of MTPI on apoB secretion from primary mouse hepatocytes. Cells were preincubated with or without MTPI for 2 hours

followed by the addition of 100 μCi/ml of [^{35}S]methionine for 4 hours. The media was collected and apoB was immunoprecipitated and analyzed by SDS-PAGE and fluorography. The results obtained confirmed the observations of Raabe et al. (Raabe *et al.*, 1999). Total apoB100 secretion into the media was reduced by ~ 70% in cells that had been administered 1μM MTPI (Fig. 3.5.). Total apoB48 secretion, on the other hand, remained the same in both control and MTPI-treated cells. However, when hepatocytes were subjected to 10 μM MTPI, the levels of apoB48 decreased by approximately 30% (Fig. 3.5) suggesting that MTP inhibition may have an effect on total apoB48 secretion as well, but the effect was only observed at higher levels of inactivation of MTP. At 10μM of MTPI, secreted apoB100 is barely detectable. Therefore, MTPI displays much greater effect on apoB100 than apoB48.

Next, we investigated the density of secreted apoB-containing particles. Hepatocytes were pulse-labeled for 4 hr with 100mCi/ml of [^{35}S]methionine, in the presence or absence of MTPI, and 0.375 mM oleate. The media was collected and placed on a sucrose gradient to separate lipid-poor from lipid-rich particles, which were then immunoprecipitated with anti-apoB antibody. In cells incubated in the absence of MTPI, apoB100 was secreted mostly with lipid-rich particles (Fig. 3.6). Little to no apoB100 was found associated with secreted lipid-poor particles. ApoB48 was associated with both, lipid-rich and lipid-poor particles (Fig. 3.6), but clearly the levels of lipid-rich apoB48 were decreased.

As expected, apoB100 association with secreted lipoproteins was too low for detection in hepatocytes treated with MTPI. The density profile of apoB48-

containing lipoproteins displayed a significant decrease in the amount of lipid-rich particles and an increase in the amount of apoB48 secreted with lipid-poor particles in comparison to the control cells.

These results imply that in order for apoB100 to enter the secretory pathway it needs to be fully lipidated; a requirement met only in the presence of MTP. For the secretion of apoB48-containing lipoproteins, MTP is not as crucial, and the presence of apoB-containing lipoproteins was still observed during MTP inhibition. However, apoB48-containing particles were lipid-poor compared to those secreted by control hepatocytes.

To illustrate further the lipidation status of all secreted particles we labeled lipids with [^{3}H]oleate for 4 hr. Medium was collected and subjected to density fractionation. Lipids were extracted from each of 12 density fractions and analyzed by TLC. Control hepatocytes secreted a significant amount of TG in the VLDL density range (Fig. 3.7). TG was also found to be secreted with lipid-poor lipoproteins, albeit to a modest extent. These results are consistent with the results for secreted apoB-containing lipoproteins where apoB100 was found to be associated mostly with VLDL (results shown in Fig. 3.6). In MTPI-treated cells however, only a small amount of TG was found in the lipid-rich particles. It is most likely that this TG is packaged within the core of apoB48-containing lipoproteins since the secretion of apoB100-containing lipoprtotens was abolished in MTPI-treated cells (shown in Fig. 3.7 - MTPI). We also investigated the effect of MTP I on the secretion of phospholipids. We labeled lipids with [^{3}H]oleate for 4 h and collected media from MTPI treated and control cells. The amount of

radioactivity of TLC resolved phospholipids was measured by densitometric scanning. It was found that the amount of phospholipids in the presence or absence of MTPI was not affected (Fig. 3.8). We concluded that inhibition of MTP has no effect on phospholipid secretion.

3.5 MTPI reduces the amount of lumenal TG that is not associated with apoB

After the immunoprecipitation of [^{3}H]oleate-labeled apoB-containing lipoproteins under non-denaturing conditions (Fig. 3.4) we further investigated the TG in the microsomal lumenal contents. There are reports suggesting that the microsomal lumen of hepatocytes (Raabe *et al.*, 1999) and enterocytes (Hamilton *et al.*, 1998) contain a TG droplet that is not associated with apoB. To avoid contamination of lumenal TG stores with cytosolic TG, we treated extracted microsomes with 0.5 M KCl to remove the cytosolic lipid droplet that may have been associated with ER membrane. Interestingly, we found a large amount of TG not associated with apoB, indicating the existence of a lumenal lipid store.

Lipids of hepatocytes incubated in the presence or absence of MTPI were labeled for 30 min as described for Fig. 3.4. Microsomes were isolated and treated with KCl, than ultracentrifuged to remove the cytosolic lipid droplet from the micorsomes. The re-isolated microsomes were then subjected to sodium carbonate extraction to obtain lumenal contents which were finally subjected to immunoprecipitation with an apoB antibody to obtain apoB-containing

lipoproteins. The recovered lipoprotein-associated TG was analyzed by TLC (as shown in Fig. 3.4) and any TG remaining in the lumenal contents was also analyzed. The immunodepletion of apoB from the lumenal contents was evaluated by Western blotting (not shown) and was found to be complete. We found that MTPI treatment resulted in a 50 % decrease in the lumenal TG store not associated with apoB (Fig. 3.9). Furthermore, the amount of labeled TG in the lumenal lipid store was approximately three-fold larger than the amount of TG isolated from apoB-containing lipoproteins enclosed within the lumen of ER (Fig. 3.10). These results suggest that MTP may assist in the transfer of TG from a cytosolic lipid droplet to a lumenal lipid droplet.

To address this possibility, we also examined the TG content of the cytosolic lipid droplet. If MTP were required for the transport of cytosolic TG into the ER lumen, an accumulation of TG in the cytosolic droplet in the presence of MTPI might be observed. Pulse labeling with [^{3}H]oleate for 1 hr followed by 4 hr chase, showed only ~10% increase in the amount of TG in the presence of MTPI (not shown), which is not statistically significant. For the accumulation to be evident, we may have to look at the secretion that occurs over a longer time-course. However, such a time course is not feasible under the culture conditions used for the primary hepatocytes in these experiments (enzymes lose their activity with time).

Fig. 3.1. **Secretion of TG decreases with increasing concentration of MTPI.** Cells were preincubated with lipid-free medium for 2 hr in the presence of increasing concentration of MTPI (0-10 μM) followed by the addition of 5 μCi/ml [^{3}H]oleate in DMEM containing 0.375 mM non-labeled oleate for 4 hr in the presence of MTPI at the indicated concentrations. The medium was collected, lipids extracted and analyzed on TLC. TLC plates were scraped and DPM in TG measured by scintillation counting. The error bars represent standard deviations of three separate experiments.

Figure 3.1

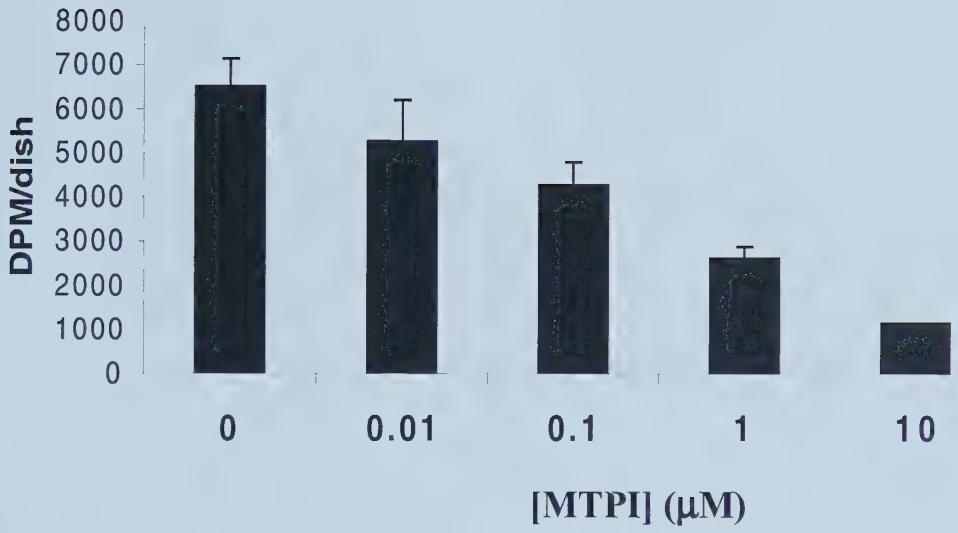
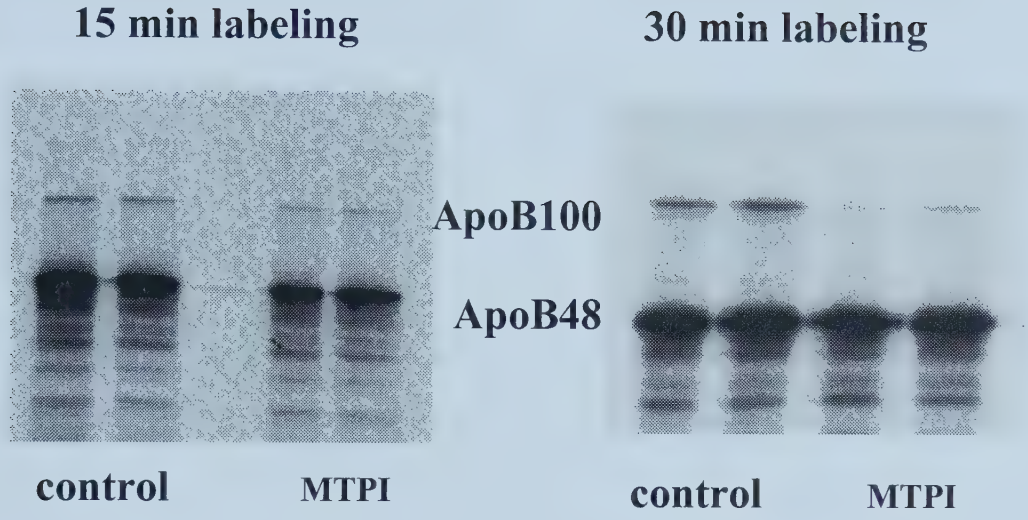

8000
7000
6000
5000
4000
3000
2000
1000
0
DPM/dish
0
0.01
0.1
1
10
[MTPI] (μM)

Fig. 3.2. **ApoB is translocated across the membrane of ER in the presence of MTPI.** Mouse hepatocytes were preincubated with methionine free medium containing 0.375 mM oleate in the presence or absence of MTPI (10μM) for 2 hr. Cells were then labeled with 100μCi/ml of [^{35}S]methionine for 15 or 30 min +/- MTPI, permeabilized with digitonin (75 μg/ml) for 5 min on ice, and trypsinized (100μg/ml) for 10 min on ice. The digitonin concentration and time it takes to permeabilized cell membrane was studied by using a lactate dehydrogenase assay, a cytosolic enzyme whose product is released upon permeabilization. The trypsin sensitivity was determined with phosphatidylethanolamine N-methyltransferase (PEMT) and cholinephosphotransferase (EPT) assays. Trypsin digestion was stopped by the addition of protease inhibitors. The remaining cellular contents were solubilized in TritonX-100-containing buffer. Protected apoB was obtained by immunoprecipitation and analyzed by SDS-PAGE and fluorography. (A) SDS-PAGE representing apoB100 and 48 immunoprecipitated from lumen and inner ER membrane labeled for 15 and 30 min. (B) Quantification of apoB100 and 48 present in the lumen and on the inner ER membrane after 15 and 30 min labeling. The numbers were obtained by densitometric scanning of the radiograph and the error bars represent standard deviations of three separate experiments. *Open bar*, control*; closed bar,* MTPI.

Figure 3.2

A


15 min labeling
30 min labeling
ApoB100
ApoB48
control
MTPI
control
MTPI

B

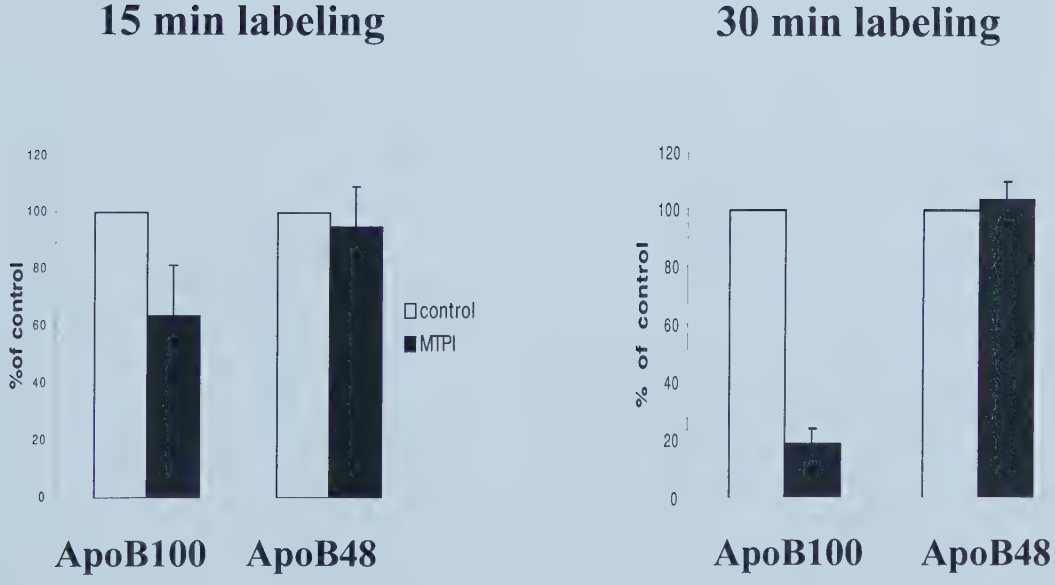
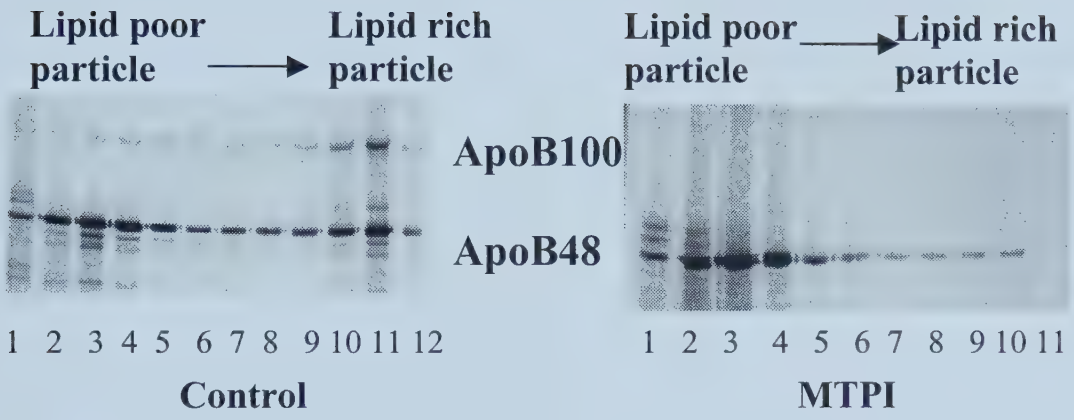

15 min labeling
30 min labeling
%of control
% of control
120
100
80
60
40
20
0
control
MTPI
ApoB100
ApoB48
ApoB100
ApoB48

Fig. 3.3. **In the presence of MTPI, apoB100 lipidation was abolished whereas apoB48 was associated only with lipid poor particles.** Mouse hepatocytes were preincubated with methionine-free medium containing 0.375 mM oleate in the presence or absence of MTPI (1μM) for 2h, labeled with 100 μCi/ml of [^{35}S]methionine for 30 min +/- MTPI, collected and homogenized with 0.25 M sucrose. Isolated microsomes were subjected to sodium carbonate extraction and then ultracentrifugation to separate lumenal contents and membranes. The supernatant containing lipoproteins was applied to a sucrose gradient and ultracentrifuged for 60 hr. Samples were collected in 12 fractions ranging from 1.006 g/ml, which is the top fraction (lipid-rich particles), through 1.21 g/ml, which is the bottom fraction (lipid-poor particles). ApoB was recovered from each fraction by immunoprecipitation and analyzed by SDS-PAGE and fluorography. (A) SDS-PAGE of immunoprecipitated apoB100 and 48 from gradient fractions of lumenal lipoproteins. (B) Quantification of (A) by densitometric scanning. The error bars represent standard deviations of three separate experiments. *Open* bar, control; *closed* bar, MTPI

Figure 3.3

A


Lipid poor particle
Lipid rich particle
Lipid poor particle
Lipid rich particle
ApoB100
ApoB48
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11
Control
MTPI

B

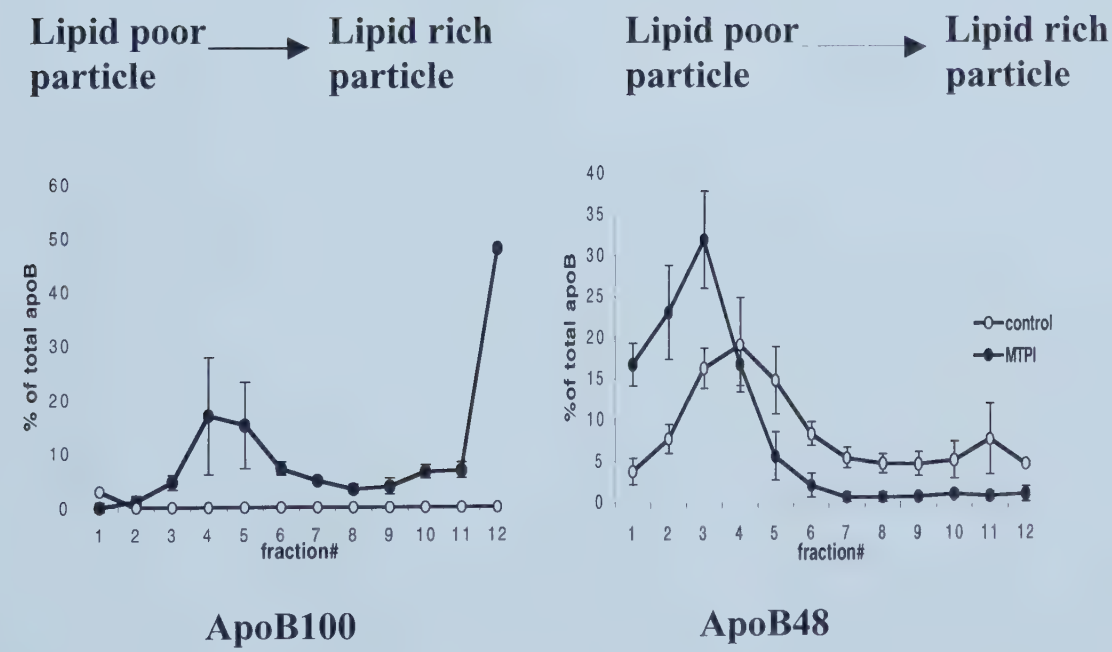

Lipid poor particle
Lipid rich particle
Lipid poor particle
Lipid rich particle
% of total apoB
fraction#
%of total apoB
control
MTPI
fraction#
ApoB100
ApoB48

Fig. 3.4. **TG content of lipoproteins in the lumen is reduced in the presence of MTPI.** Hepatocytes were preincubated with lipid-free medium for 2 hr in the presence or absence of MTPI (10μM) followed by the addition of 10μCi/ml [^{3}H]oleate in DMEM containing 0.375 mM cold oleate, +/-MTPI for 30 min. Cells were collected and homogenized with sucrose. Microsomes were obtained and subjected to sodium carbonate extraction. Broken membranes were pelleted and supernatants containing lumenal contents were subjected to immunoprecipitation under non-denaturing conditions to obtain apoB-containing lipoproteins. Lipids were extracted from the lipoproteins then analyzed by TLC, scraped and measured by liquid scintillation counting. TG isolated from lumenal lipoproteins is shown as a percent of control (values for control cells are ~1000 DPM) and the error bars are standard deviations of three separate experiments.. *Open bar*, control*; closed bar,* MTPI.

Figure 3.4

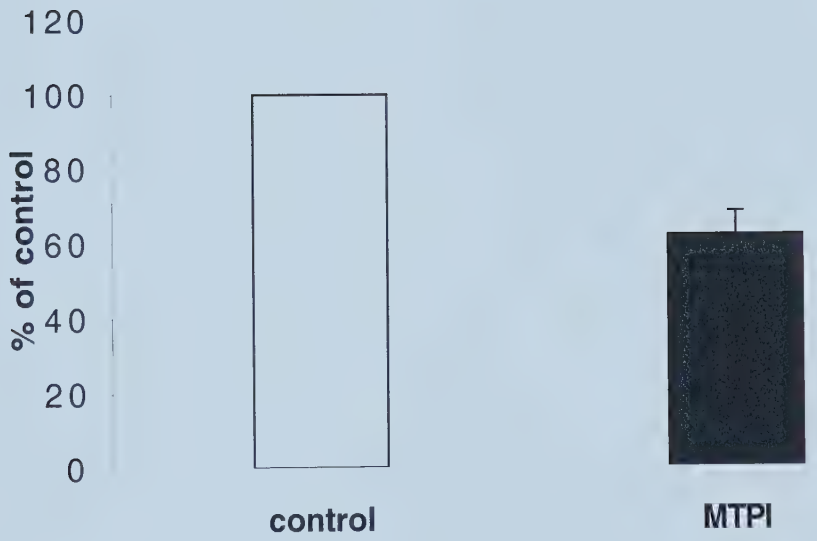
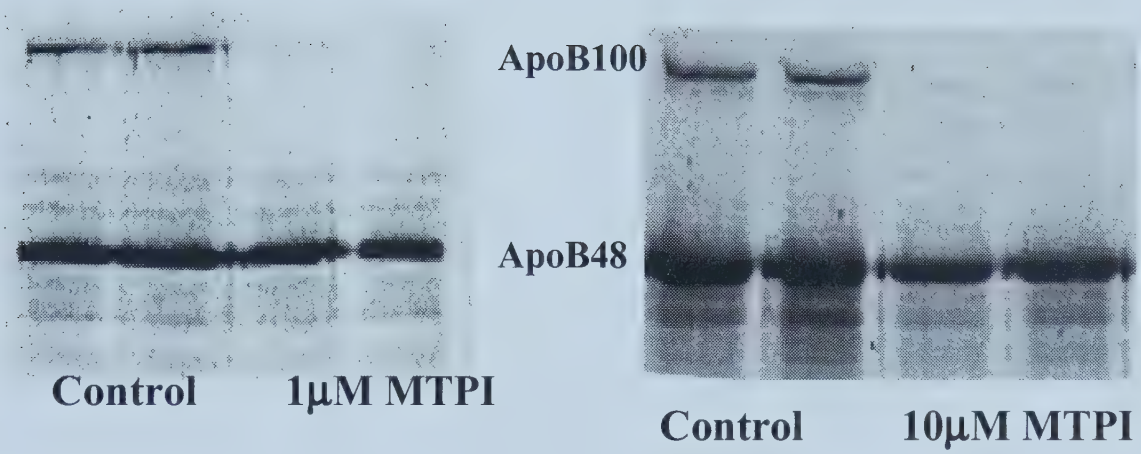


Fig. 3.5. **Total apoB secretion decreases with increasing concentration of MTPI.** Mouse hepatocytes were preincubated with methionine free medium containing 0.375 mM oleate in the presence or absence of MTPI (1 or 10 μM, as indicated) for 2 hr. Cells were then labeled with 100μCi/ml of [^{35}S]methionine for 4 hr, medium collected and apoB immunoprecipitated. The levels of [^{35}S]apoB secretion were analyzed by SDS-PAGE and fluorography and apoB protein levels measured with densitometric scanning. (A) SDS-PAGE representing immunoprecipitated apoB100 and apoB48 from the medium of cells treated with 1 and 10 μM MTPI. (B) Quantification of secreted apoB from panel A presented as a percentage of control apoB. The error bars represent standard deviations of three experiments for 1μM MTPI and two experiments for 10 μM MTPI. *Open bar*, control; *closed bar,* MTPI.

Figure 3.5

A


ApoB100
ApoB48
Control
1μM MTPI
Control
10μM MTPI

B

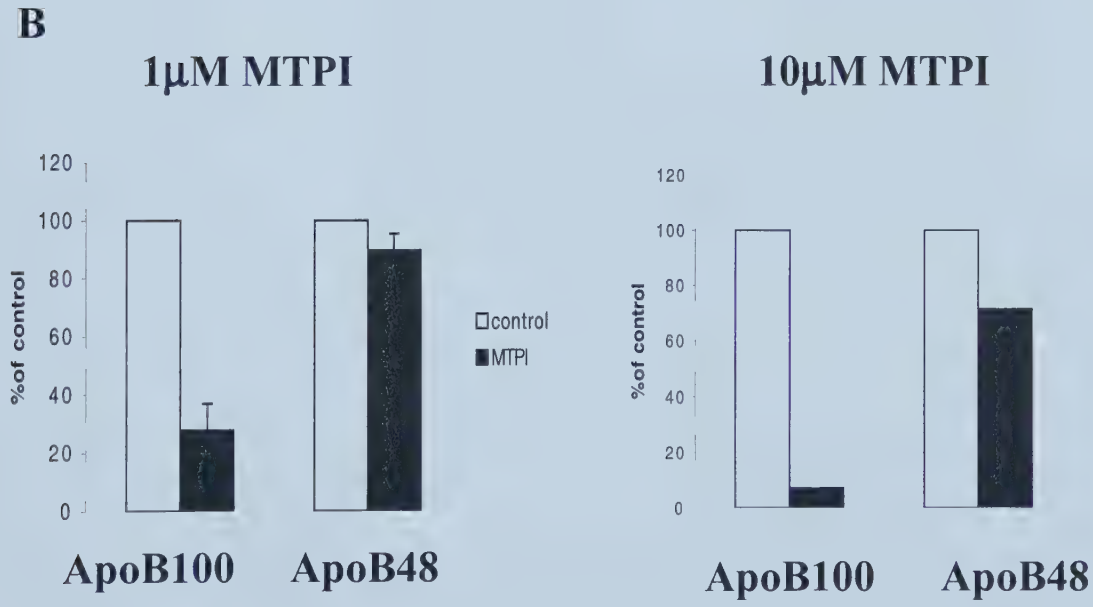
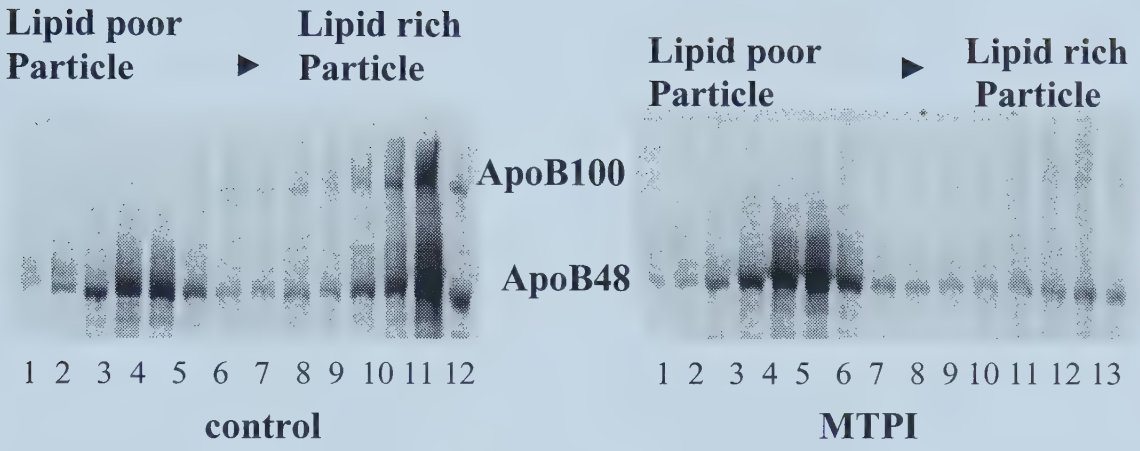

1μM MTPI
10μM MTPI
%of control
120
100
80
60
40
20
0
control
MTPI
ApoB100
ApoB48
ApoB100
ApoB48

Fig. 3.6. **ApoB100 secretion is abolished whereas apoB48 is secreted only on lipid-poor particle in cells treated with MTPI.** Mouse hepatocytes were preincubated with methionine-free medium containing 0.375 mM oleate in the presence of MTPI (1μM) for 2 hr. Cells were then labeled with 100μCi/ml of [^{35}S]methionine for 4 hr, medium collected, applied to a sucrose gradient and ultracentrifuged for 60 hr. Samples were collected in 12 fractions ranging from 1.006 g/ml, which is the top fraction (lipid-rich particles), through 1.21 g/ml, which is the bottom fraction (lipid-poor particles). The levels of apoB secretion were analyzed by SDS-PAGE and fluorography (panel A) and the data quantified by densitometric scanning (panel B) (AU=arbitrary units). This experiment is representative of three separate experiments. *Open* symbols, control; *closed* symbols, MTPI.

Figure 3.6

A


Lipid poor Particle
Lipid rich Particle
ApoB100
ApoB48
1 2 3 4 5 6 7 8 9 10 11 12
control
Lipid poor Particle
Lipid rich Particle
1 2 3 4 5 6 7 8 9 10 11 12 13
MTPI

B

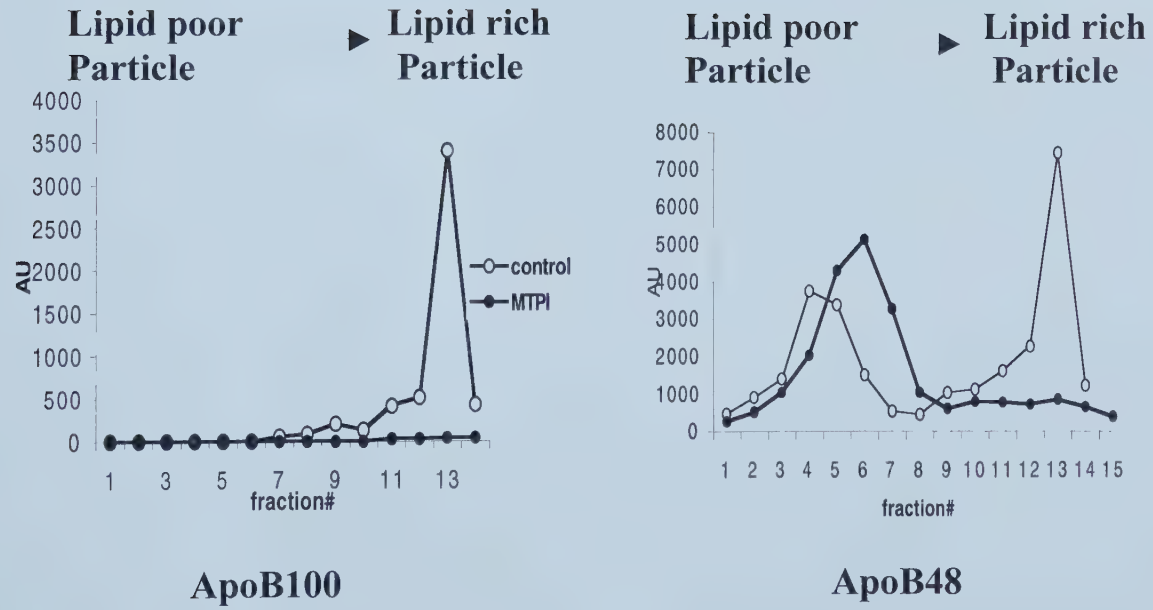

Lipid poor Particle
Lipid rich Particle
AU
4000
3500
3000
2500
2000
1500
1000
500
0
1 3 5 7 9 11 13
fraction#
control
MTPI
ApoB100
Lipid poor Particle
Lipid rich Particle
AU
8000
7000
6000
5000
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
fraction#
ApoB48

Fig. 3.7. **Secretion of VLDL-TG is diminished in the presence of MTPI.** Cells were preincubated with medium free of lipids for 2 hr in the presence or absence of MTPI (1 μM), followed by the addition of 5 μCi/ml [^{3}H]oleate in DMEM containing 0.375 mM unlabeled oleate for 4 hr in the presence or absence of MTPI. The medium was collected, placed on a sucrose gradient and ultracentrifuged for 60 hr. Samples were collected in 12 fractions ranging from 1.006 g/ml, which is the top fraction (lipid-rich particles), through 1.21 g/ml, which is the bottom fraction (lipid-poor particles). TG was extracted from each fraction and analyzed by TLC and measured by scintillation counting. The error bars represent standard deviations of three separate experiments. *Open* symbols, control; *closed* symbols, MTPI.

Figure 3.7

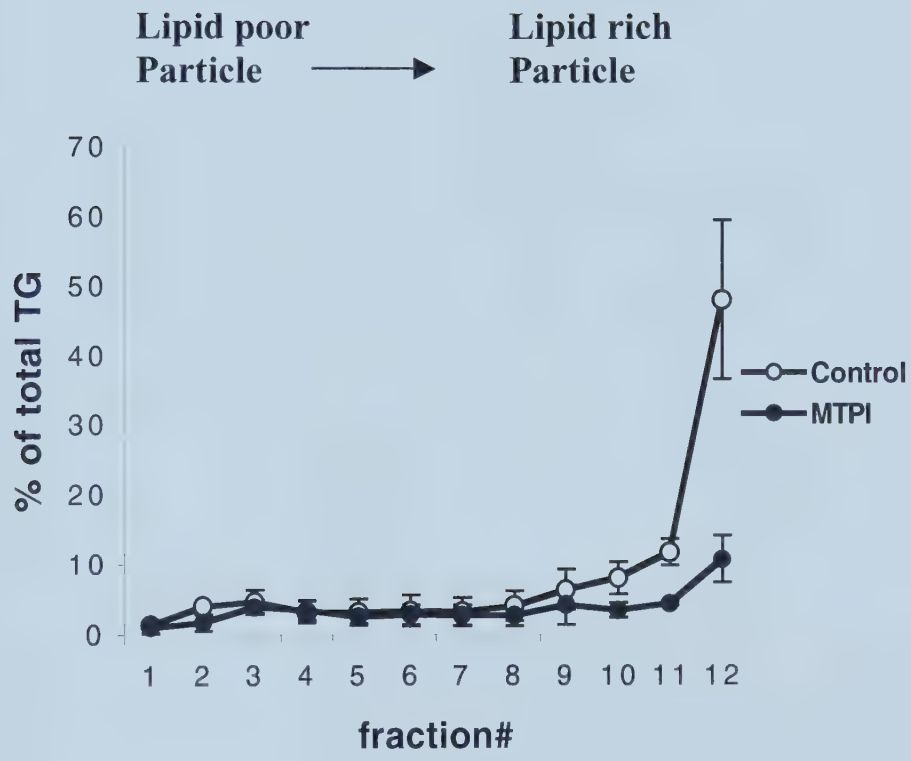

Lipid poor
Particle
Lipid rich
Particle
% of total TG
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12
fraction#
Control
MTPI

Fig. 3.8. **Secretion of PL is unaffected by the presence of MTPI.** Cells were preincubated with lipid-free medium for 2 hr in the presence or absence of 10 μM MTPI followed by the addition of 5 μCi/ml [^{3}H]oleate in DMEM containing 0.375 mM non-labeled oleate for 4 hr in the +/- MTPI. The medium was collected, lipids extracted and analyzed on TLC. TLC plates were scraped and DPM measured by scintillation counting. The graph is representative of three separate experiments and the error bars are standard deviations of three samples.

Figure 3.8

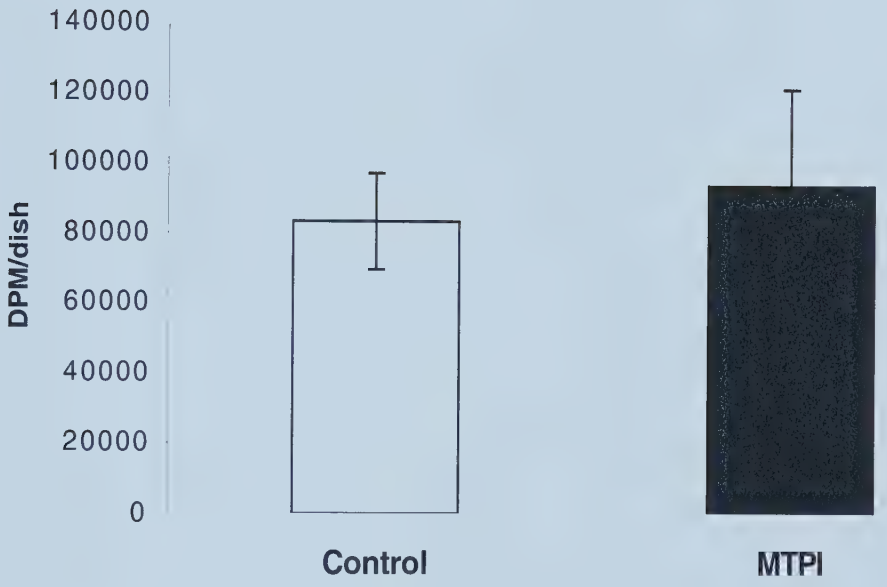

140000
120000
100000
80000
60000
40000
20000
0
DPM/dish
Control
MTPI

Fig. 3.9. **The amount of TG isolated from lumenal lipid droplet in the presence of MTPI.** Hepatocytes were preincubated with medium free of lipids for 2 hr in the presence or absence of MTPI (10μM) followed by the addition of 10μCi/ml [^{3}H]oleate in DMEM containing 0.375 mM cold oleate, +/-MTPI for 30 min. Cells were collected homogenized with sucrose and obtained microsomes treated with 0.5 M KCl to remove attached cytosolic TG droplet and ultracentrifuged to pellet microsomes. Microsomes were then subjected to sodium carbonate extraction, broken membranes pelleted and supernatant containing lumenal contents was used to immunoprecipitate apoB-containing lipoproteins under non-denaturing conditions. The remaining lumenal contents were analyzed for the presence of lipids by TLC and measured by scintillation counting. TG isolated from lumenal lipid droplet are shown as a percent of control. and the error bars represent standard deviations of three separate experiments.

Figure 3.9

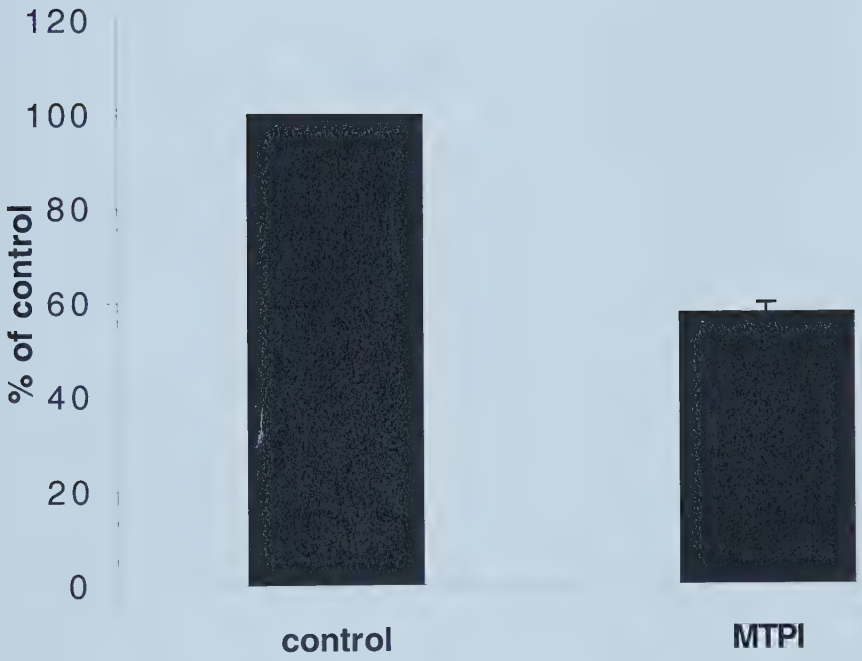
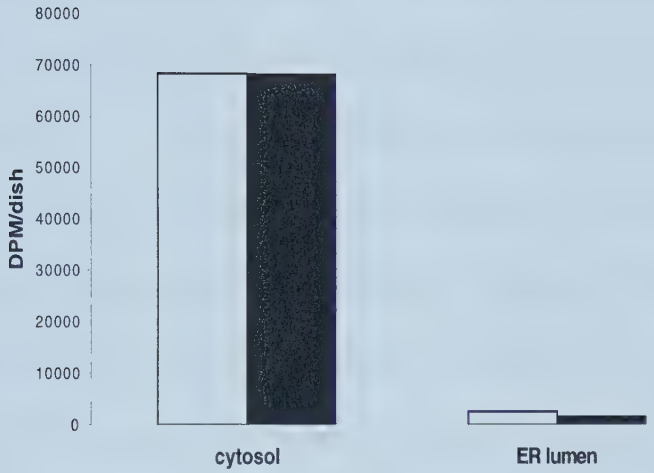


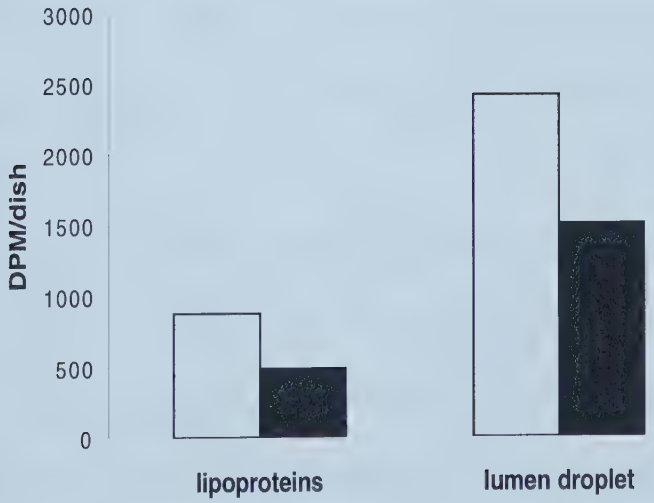
Fig. 3.10. **Distribution of TG among cytosolic lipid stores, lumenal apoB-containing lipoproteins and lumenal lipid droplet in the presence or absence of MTPI.** Hepatocytes were preincubated with medium free of lipids for 2 hr in the presence or absence of MTPI (10μM) followed by the addition μCi/ml [^{3}H]oleate in DMEM containing 0.375 mM cold oleate, +/-MTPI for 30 min. Cells were collected homogenized with sucrose and obtained microsomes treated with 0.5 M KCl to remove attached cytosolic droplet and ultracentrifuged to pellet microsomes. Microsomes were then subjected to sodium carbonate extraction, broken membranes pelleted and supernatant containing lumenal contents was used to immunoprecipitate apoB-containing lipoproteins under non-denaturing conditions. The remaining lumenal contents and cytosolic lipids were analyzed for the presence of lipids by TLC and measured by scintillation counting. (A) TG obtained from cytosolic lipid droplet and ER lumen in the presence or absence of MTPI. (B) TG recovered from lumenal apoB-containing lipoproteins and lipid droplet located in the lumen of ER. The graphs are representative of three separate experiments. *Open bar*, control*; closed bar,* MTPI.

Figure 3.10

A



B



Chapter 4 - Discussion

4.1 10 μM of MTPI inhibits ~80% TG secretion

The purpose of this study was to elucidate the mechanisms of action of MTP. This process has been extensively examined and the results obtained by different investigators are highly controversial and differ according to the cell line and methodology used. There are many theories put forward that suggest the mechanisms of action of MTP. In one, MTP functions as a chaperone to bring apoB across the ER membrane and into the lumen (Linnik & Herscovitz, 1998). The second theory was based on its *in vitro* action and suggests that MTP transfers lipids from the membrane of ER to apoB as shown in figure 1.6 (Read *et al.*, 2000), (Nicodeme *et al.*, 1999). Some studies argue that MTP delivers a bulk of lipids to a poorly-lipidated particle of high density within the lumen of the ER (Wang *et al.*, 1997b). A recent theory implies that MTP is involved in mobilizing lipids from the cytosolic lipid droplet into the lumen of ER (Raabe *et al.*, 1999).

The work presented here was performed in primary mouse hepatocytes, a cell model infrequently used to study apoB-containing lipoprotein assembly. These cells bear a greater resemblance to human hepatocytes than observed in HepG2 or McA-RH7777 carcinoma cell lines where expression of many enzymes involved in lipid metabolism and the assembly and secretion of VLDL are absent or impaired (Blackhart *et al.*, 1990), (Dashti *et al.*, 1987). Hepatocytes from Syrian hamsters behave a lot like human hepatocytes with regards to the assembly

and secretion of VLDL. We studied VLDL assembly in mouse hepatocytes since not much is known about this process in this animal model and many knock out mice of genes related to lipid metabolism are created. Furthermore, the development of liver specific and conditional MTP "knock out" mouse in Stephen Young's laboratory (Raabe *et al.*, 1999) provided us with collaborative opportunity to study the effect of the absence of MTP on the assembly of VLDL. First, we used primary hepatocytes obtained from wild type mice, administered the inhibitor to MTP and looked at the effects on the VLDL assembly. Next, we planned to compare the findings to those in primary hepatocytes from MTP KO mice. Unfortunately, in our hands, we could only achieve 15% success (3 successful knockouts out of 20) in conditionally knocking out the MTP gene. Because of the low number of successful "knock outs" and the large degree of variability between $MTP^{-/-}$ mice we were unable to gain confident conclusions from the results we produced. Therefore, only the findings from hepatocytes of wild type mice in the presence or absence of an MTP inhibitor are presented.

First, we determined the concentration of BMS-197636, an MTP inhibitor that would successfully inhibit the action of MTP. A common approach to test the inhibition of MTP activity is to perform an *in vitro* test that involves transfer of labelled lipids from donor SUV to the acceptor SUV in the presence or absence of MTP inhibitor (this approach is described in the introduction). However, this test does not take into the consideration the physiological obstacles the cell creates and the actual time it takes for the inhibitor to reach its target. Furthermore, the concentration of inhibitor it takes to reach the target may be

different for cells in culture. To approximate the inhibition of MTP we measured the amount of TG secreted in the presence of the inhibitor. The inhibitor concentration found to decrease between 80 and 90 % of TG secretion was 10 μM. The degree of inhibition was dependent on the strain of mouse we used. For example, Balbc mice were more resistant to MTPI than C57b/6 mice, and to conserve the inhibitor we chose to use C57b/6. Some variability was also observed from animal to animal within the same strain. However, the differences were not as substantial as between two different strains.

4.2 MTP does not chaperone apoB across the ER membrane

The first experiment was performed to address the question of whether or not MTP chaperone activity was required for VLDL assembly. We showed (Fig. 3.2) that in mouse primary hepatocytes MTP is not required for the translocation of apoB across the ER membrane. Both apoB100 and apoB48 were found protected by the microsomal membrane in MTPI-treated and control cells when assessed by a trypsin digestion assay. The results, however, did not distinguish whether apoB entered the microsomal lumen or was inserted into the inner leaflet of ER membrane. After 30 min of labelling, the amount of apoB 48 that was protected from trypsin digestion increased independently of the presence or absence of MTPI (Fig. 3.2). By contrast, in MTPI-treated cells compared to control cells, the levels of apoB100 found in the microsomes were decreased significantly, whereas the amount of apoB100 in control cells increased with time.

The reduction of apoB100 in MTPI treated cells cannot be attributed to secretion since 30 min is not long enough for apoB100 to enter the secretion pathway (Bostrom *et al.,* 1986). This suggests that apoB100 was degraded. The results presented in Fig. 3.3 show that normal amounts of apoB48 entered the lumen of ER in the presence of MTPI, however we could not show the same for apoB100 even though we used proteosome inhibitors which turned out to be toxic to our hepatocytes but were successful in other cell lines (Benoist & Grand-Perret, 1997). Currently, it is not clear if the proteosome pathway for apoB degradation occurs in primary hepatocytes as it does in HepG2 cells. The studies in HepG2 cells treated with the proteosome inhibitors and MTPI demonstrated that a large amount of synthesized apoB accumulated in the lumen of ER. ApoB that was misfolded due to the MTPI and that would normally enter the degradation pathway was rescued, but remained secretion incompetent. Thus, the secretion of apoB remained unchanged in comparison to cells incubated in the absence of proteosomal inhibitors (Benoist & Grand-Perret, 1997). The misfolded apoB that is normally degraded in the presence of proteosomal inhibitor, is secretion incompetent when MTP is inhibited and thus may build up in the ER. The reason why we saw apoB48 but not apoB100 in the lumen may be explained by the difference in acquiring the proper conformation as a function of lipid availability, which is reduced in the presence of MTPI. In fact, it has been implied that the presence of lipids allows apoB to assume the proper conformation, rescuing it from the degradation pathway (Ingram & Shelness, 1997). Because apoB100 is significantly larger than apoB48, it requires more lipids to obtain a suitable

conformation. We suggest that the inhibition of MTP reduces the lipid availability and the subsequent lipidation process, which is more evident for apoB100 since it needs a larger number of lipids to escape the degradation pathway than does apoB48 (apoB48 can be found associated with phospholipids alone(ref)).

4.3. MTPI reduces addition of lipids to apoB

Interestingly, we recovered some apoB48 lipid-rich particles from the lumen of ER (Fig. 3.3), suggesting that it is less favourable for the cell to assemble apoB100 lipid poor particles than apoB48 lipid-rich particle. One explanation is that the assembly of apoB48-containing lipoproteins might occur faster than apoB100-containing lipoproteins and when MTP is inhibited the available lipids are used up quickly, even before apoB100 gets a chance to gain enough lipids to escape degradation and to enter the lumen of ER.

TG isolated from apoB-containing lipoproteins enclosed by the microsomal lumen of MTPI treated cells was decreased by about 40% compared to control cells (Fig. 3.4), which was also consistent with the decrease in apoB (Fig. 3.3). These results suggest that MTP inhibition affected the addition of TG to the lipoproteins. However, the amount of phospholipids associated with lipoproteins remained unchanged in the presence of the inhibitor (Fig. 3.8). This suggests the MTPI does not influence the phospholipid transfer or the association

of apoB with phospholipids is independent of MTP. In addition, the recovered TG was likely associated with apoB48 since only small amount of apoB100 was found to enter the lumen of ER.

4.4 Secretion and lipidation of apoB-containig lipoproteins is greatly inhibited by the increasing concentration of MTPI.

Next we looked at the effects of MTPI on the secretion of apoB and lipids. As expected, apoB100 secretion was significantly reduced (~90%) but the secretion of apoB48 was not affected. Only as the concentration of the MTPI was increased, its consequence on apoB48 secretion became more apparent. This suggests that the higher the concentration of MTPI the greater the effect on MTP lipid transfer, thus less lipid is available to apoB48 to acquire a proper conformation and to escape the degradative processes. Our results suggest that both full-length apoB and apoB48 are affected by MTPI. However, the effect is more prominent with apoB100 possibly due to a larger amount of lipid required by apoB100 to assume a proper conformation that would permit further lipidation rather than retrograde transport and ultimately degradation.

Density fractionation of secreted apoB-containing lipoproteins showed that cells treated with MTPI secreted no detectable apoB100. On the other hand, the profile of apoB48 secretion was shifted in favour of lipid-poor particles as a result of MTPI treatment. The secretion of some apoB48 with lipid-rich lipoproteins in the presence of MTPI is likely due to incomplete inhibition of

MTP (~80%). The overall absence of apoB100 secretion from MTPI treated cells is likely due to insufficient lipidation and hence retrograde transport and degradation.

4.5. MTPI inhibits transfer of TG to the lumenal lipid droplet

Newly synthesized apoB48 associates with phospholipids (a process that appears independent of MTP), enters the lumen, uses up the available lipids, and then is secreted. Density fractionation of secreted lipids showed a large decrease in TG in lipid-rich fractions in the presence of MTPI. Clearly, the interpretation is that these lipid-rich fractions contain fewer TG-rich lipoprotein particles and residual MTP activity is limited to optimal lipidation of only apoB48.

The isolation of the lumenal lipid droplet provided yet another novel and interesting level of insight on what may be happening when MTP is inhibited. We found TG in the lumen of ER that was not associated with apoB. The detected TG was not due to the contamination from the cytosol because of the KCl treatment, which ensures the removal of any attached cytosolic TG droplet from the ER membrane. The presence of MTPI reduced the amount of TG in the lumenal lipid stores by about 50% (Fig. 3.9). The results suggest that MTP is involved in the mobilization of TG from the cytosolic lipid droplet, into the lumen. This is further supported by the observation that the TG of carbonate-extracted lumenal lipoproteins is also decreased by approximately 50%. Furthermore, the results suggested that the lumenal lipid droplet may be a primary

acceptor of TG, since more labeled TG (approximately three-fold) was found in the lumenal stores than in apoB-containing lipoproteins within the same compartment (Fig. 3.10 B). The participation of MTP in the transfer of lipids between lumenal droplet and lipoproteins still needs to be addressed. However, since the lumenal lipid droplet is partially depleted upon MTP treatment, MTP may participate in the movement of lipids, either by addition of individual lipid molecules or by fusion with a lipid droplet (Gordon *et al.*, 1996), between lipoproteins being assembled in the ER and the ER lumenal TG store. However, as suggested previously, the joining process with lipid-poor particles and the lipid droplets may be MTP independent (Gordon *et al.*, 1996). If the number of lumenal droplets is decreased, then the occurrence of a lipid-poor lipoprotein "bumping" into a lipid droplet will lessen as well. At least one theory put forth suggests that *in vitro* at least, MTP transfers lipid monomers between membrane vesicles (Atzel & Wetterau, 1993).

A recent study implied that MTP might play a critical role in the movement of TG into the lumen of the ER. Targeted disruption of the MTP gene in murine liver resulted in the absence of VLDL particles within either the ER or Golgi apparatus and numerous cytosolic lipid droplets (Raabe et al., 1999), as determined by electron microscopy. In a separate study, rabbits treated with MTPI displayed an accumulation of TG in the cytosol with prolonged MTP inactivation (Wetterau e*t al.*, 1998 #48). Wang *et al.* had postulated that MTP may be an important protein factor in the mobilization of TG into the microsomal lumen since MTP inactivation inhibits TG secretion, yet it does not inhibit TG

synthesis (Wang *et al.*, 1999). They rationalized that the decreased influx of TG from the cytosolic droplet may be the most likely explanation for diminished lumenal TG content as measured by decreased secretion. In primary mouse hepatocytes treated with MTPI we did not observe a significant increase in cytosolic TG (only ~10%) possibly because of the enormous size of the cytosolic TG pool so that any increase in TG would be insignificant. Furthermore, TG required for lipoprotein assembly can also be obtained from *de novo* synthesis (Glaumann *et al.*, 1975). A major challenge to measure the effect of MTPI on the cytosolic TG droplet is the short life span of cultured primary hepatocytes.

In mice that were deficient in intestinal apoB synthesis, a vast number of large lipid "particles" (2- to 3-fold larger than those in the intestinal cells of control mice) were found in the lumen of enterocytes, fewer lipid-staining particles in the Golgi apparatus, and even fewer in the extra-cellular space (Hamilton *et al.*, 1998). The study concluded that chylomicron formation might involve the synthesis of apoB-free TG-rich particles within the ER lumen. These results suggest that TG is mobilized most likely by MTP and enters the lumen regardless of the presence or absence of apoB.

4.6. A model for the mechanisms of action of MTP

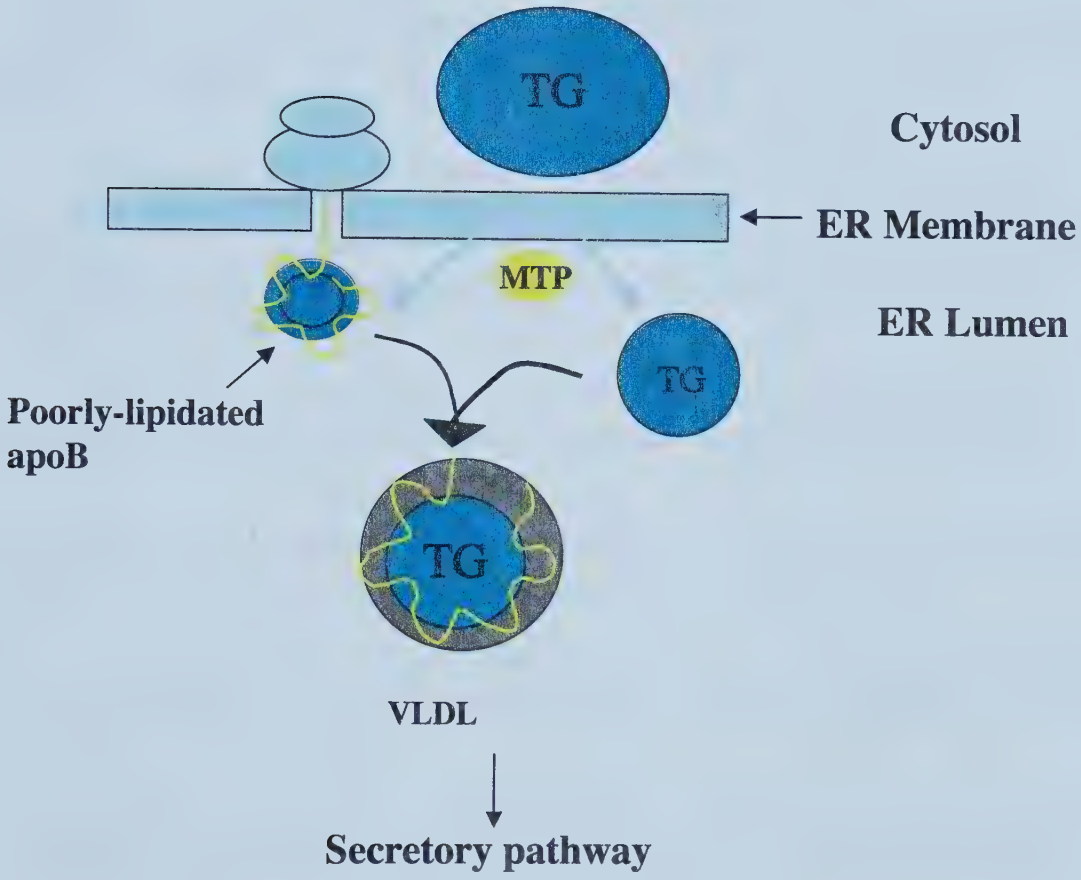
Our results allowed to propose a model for the mechanisms of action of MTP and its role in the assembly of apoB-containing lipoproteins (Fig. 4.1). In primary hepatocytes, TG is stored in the cytosol in the form of lipid droplets that

are localized in the proximity of the ER membrane. TG contained within those stores has to be mobilized for the assembly of VLDL, which involves the transfer of TG fatty acids across the phospholipid bilayer of the ER in order to congregate with apoB. This process takes place at the ER membrane and involves cytosolic TG lipolysis and re-esterification of the resultant fatty acids to TG. We propose that the TG acceptor is MTP which inserts itself into the lipid membrane via a "fusion peptide" promoting interactions with lipids and flux into the hydrophobic lipid binding cavity (Read *et al.*, 2000). When MTP attains enough lipids (up to five TG molecules (Atzel & Wetterau, 1993)) it detaches from the membrane and transfers them to either apoB or to a lumenal lipid droplet. For MTP to bind to apoB, apoB needs a N-terminal domain properly folded, and bound phospholipids. It has been shown that sequences of apoB between apoB29 and apoB32.5 bind phospholipids, a prerequisite for TG binding (Carraway *et al.*, 2000). Phospholipids bound to apoB may attract MTP's "fusion peptide" forming an apoB-MTP complex (Read *et al.*, 2000) with an intermediate containing a "lipid pocket" (Segrest *et al.*, 1999) which accepts more lipid either by addition of individual lipids or by the fusion with a lumenal lipid droplet. MTP may also transfer lipids to the lumenal lipid droplet, which are most likely made of monolayer of phosopholipids surrounding a TG core where the hydrophobic forces facilitate the interaction and lipid transfer.

The purpose of lumenal lipid droplets needs to be elucidated, but it is likely they are comparable with cytosolic lipid droplets in respect to proteins that are associated with the drplets. The process of TG addition to preformed lipid-

poor particles may not be facilitated by MTP but may simply occur by apoB-lipid-poor particle colliding with the lipid droplet (Alexander *et al.*, 1976). This model suggests that the mechanisms of action of MTP may not be as specific as was thought and that MTP may move freely between different hydrophobic structures that are capable of accepting and delivery of lipids.

Assembly of Lipoproteins



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